OBSERVATIONS ON THE IMMUNE RESPONSES OF SHEEP INFECTED WITH *CYTOECETES PHAGOCYTOPHILA*

ZERAI WOLDEHIWET, D.V.M., M.R.C.V.S.

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DECLARATION

This thesis has been composed by me

and describes my own work

Z. Woldehiwet

ABSTRACT

Humoral and cell-mediated immune responses of sheep infected with Cytoecetes phagocytophila, the causative agent of tick-borne fever, were studied. Previous immunological studies were hampered by lack of reliable sources of antigen. Attempts, therefore, were made to improve antigen yields. Two methods were used successfully. The first was based on the properties of corticosteroids to induce granulocytosis; sheep injected with betamethasone sodium phosphate injection BP at the peak period of parasitaemia had a neutrophilia with an increase of infected cells within six hours. The second method involved the culture of infected blood overnight at 37°C which increased the number of infected cells and the number of organisms per infected cell.

A relationship between complement fixing antibodies and protective immunity was found and a threshold of protective immunity established. The kinetics of antibody responses to the organism were studied by fractionating immunoglobulins of sera from immune sheep by gel-filtration and ion-exchange chromatography and by complement fixation test. Antibody response to C. phagocytophila was characterised by an initial production of IgM followed by IgG but the IgM persisted for long periods.

The complement fixation test was also used to assess the antigenic relationships between strains of C. phagocytophila. All strains tested showed strong antigenic relationships but quantitative differentiation was possible.

A hitherto undescribed *in vitro* test for cell-mediated immune response of sheep was developed. The cell-mediated immune response appeared earlier than the humoral immune response.

LIST OF ABBREVIATIONS AND ACRONYMS

C	Celsius
CF	complement fixing
CFT	complement fixation test
CMI	cell-mediated immunity
DDW	deionized distilled water
DE-52	diethylaminoethylcellulose
DMSO	dimethyl sulphoxide
DTH	delayed type hypersensitivity
EDTA	dipotassium ethylenediamine tetra acetic acid
e	standard error
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
g	acceleration due to gravity
g	gram
HBSS	Hanks's balanced salt solution
ID_{50}	50 percent infective dose
IFAT	indirect immunofluorescent test
IgG	G class immunoglobulin
IgM	M class immunoglobulin
IHT	indirect haemagglutination test
IU	international units
l	litre
LMI	leucocyte migration inhibition
LMIF	leucocyte migration inhibition factor
LS	lamb serum

mg	milligram
MI	migration index
ml	millilitre
MEM	minimum essential medium
mm^2	square millimetre
mm^3	millimetre cube
μ	micron
μg	microgram
NBCS	newborn calf serum
nm	nanometre
PBS	phosphate buffered saline
PE	peritoneal exudate
pH	logarithmic index of the hydrogen ion concentration
PMN	polymorphonuclear leucocytes
RPMI	Roswell Park Memorial Institute
sd	standard deviation
STV	saline-trypsin-versane
TBF	tick-borne fever
TRIS-HCl	Tris hydroxymethyl ethylamine in hydrochloric acid
VB	veronal buffer

INTRODUCTION

The existence of tick-borne fever was not known until MacLeod (1932) accidentally discovered it in sheep grazing tick-infested pastures of Scotland. Since then it has been recognised as a disease of sheep and cattle in Europe, Asia and Africa (Tuomi, 1966; Neitz, 1969). Diagnosis of tick-borne fever (TBF) is relatively easy. The characteristically high febrile reaction is accompanied by the appearance of typical inclusions in the cytoplasms of granulocytes and monocytes which stain well with Romanowsky stains (Foggie, 1951).

Although the existence of some degree of immunity was suspected early in the history of the disease (Gordon, Brownlee, Wilson and MacLeod, 1932), our knowledge of the immune status of animals which had experienced infection with TBF is, at best, speculative. The main problem has been the lack of reproducible immunological methods (Tuomi, 1967[d]). The apparent existence of a carrier state has also contributed to the problem. Consequently the published information conflicts. Some workers claimed that immunity to TBF was solid while others reported that it was short-lived (Hudson, 1950; Stamp and Watt, 1950). Some have claimed that immunity to TBF depended on premunition or a carrier state (Foggie, 1951) while others have allegedly demonstrated immunity without the apparent presence of a carrier state (Tuomi, 1967[a]).

Another contributory factor to the confusion has been the apparent presence of various strains, even in closely located farms (Foggie, 1951). Tuomi (1967[c]), for example, claimed that the

extent of immunological heterogenity of Cytoecetes phagocytophila might be unparallelled in any other species of micro-organisms.

Although Tuomi (1967[d]) described a direct fluorescent antibody test and Snodgrass and Ramachandran (1971) a complement fixation test, no further studies on the persistence of humoral antibodies and their role on protective immunity have been reported. The purpose of the present work is to study the immune responses of sheep experimentally infected with C. phagocytophila.

CHAPTER ONE

REVIEW OF THE LITERATURE ON TICK-BORNE FEVER

HISTORICAL BACKGROUND

In an experiment initially designed to investigate the transmission of louping ill by ticks, MacLeod (1932) discovered that the tick, Ixodes ricinus was apparently harbouring an agent different from that which caused louping ill. He reproduced the disease in other sheep by sub-inoculating blood from infected sheep. Subsequently, Gordon and his colleagues (1932) described the disease after an extensive study. After immunizing some sheep against louping ill and others against the agent discovered by MacLeod (1932) they allowed them to graze on tick-infested farms. Those immunized against louping ill developed a febrile disease. They thus demonstrated that sheep immunized with louping-ill virus developed a disease caused by an agent immunologically distinct from louping-ill virus. They named the new disease "tick-borne fever". It was fortunate for the study of the disease that there was only one strain in the pastures they were investigating for it was later discovered that many strains of the disease could be found in nearby areas (Foggie, 1951).

Further studies at the Moredun Institute, Scotland, clarified many aspects of the disease. Gordon, Brownlee and Wilson (1940) reported that the causative agent was found in the cytoplasm of granular leucocytes and monocytes of infected sheep. Taylor, Holman and Gordon (1941) studied the haematological disorders that accompany the disease. Foggie (1951) elaborated the nature of the causative agent and made important contributions to the understanding of immunity against the disease.

Hudson (1950) found that the disease also affected cattle in England and Tuomi (1966) established that what was commonly known as pasture fever in Finland was in fact TBF of cattle.

Two recent advances are the ultrastructural findings of Tuomi and Von Bonsdorff (1966) and the development of the complement fixation test by Snodgrass and Ramachandran (1971).

EPIDEMIOLOGY

Geographical Distribution

The disease was first recognised in Scotland. It was later reported from other parts of the United Kingdom (Hudson, 1950; Tutt and Loving, 1955; Venn and Woodford, 1956). The disease was also discovered in Norway (Overas, 1959), the Netherlands (Bool and Reinders, 1964), Finland (Tuomi, 1966), Ireland (Colins, Hannan, Fergusson and Wilson, 1970) and Austria (Hinaidy, 1973). Outside Europe, it was reported from India (Raghavachari and Reddy, 1959) and from South Africa (Neitz, 1969).

Host Range

Natural hosts. The disease naturally affects sheep (Gordon et al., 1932) and cattle (Hudson, 1950). A significant epidemiological finding was the fact that the disease naturally affects some wildlife. Foggie (1962[a]) isolated the organism from a red deer shot on the Isle of Rhum. McDiarmid (1965) reported that he detected the organism from fallow and roe deer in New Forest in South England and Greig (1969) found that feral goats removed from forestry land of New Galloway, South-west Scotland, harboured the

organism in their blood.

Experimental hosts. Tick-borne fever has been transmitted experimentally to the goat (MacLeod and Gordon, 1933). Attempts to transmit the disease to rabbits (Hudson, 1950; Foggie, 1951), ferrets (Hudson, 1950), mice (Gordon et al., 1932; Foggie, 1951; Tuomi, 1967[a]), pigs, horses and elk (Tuomi, 1967[a]) were unsuccessful. Foggie and Hood (1961) reported that they transmitted it to splenectomized guinea-pigs and mice but there is some evidence to suggest that guinea pigs and mice naturally harbour organisms similar to C. phagocytophila. When mice and guinea pigs were splenectomized these organisms were reported to be found in abundance in the leucocytes (Snodgrass, 1974). Thus the organisms seen by Foggie and Hood (1961) might not necessarily be C. phagocytophila but some naturally occurring organisms of rodents such as that described by Tyzzer (1938) in the leucocytes of the vole.

Transmission

There is little doubt that the disease is transmitted by the tick Ixodes ricinus. MacLeod and Gordon (1933) successfully repeated MacLeod's (1932) original experiment by transmitting the disease to susceptible sheep from ticks collected from pastures and from ticks which had engorged, on their previous stage, on sheep affected by TBF. Evidence was later obtained that the causal agent was transmitted trans-stadially (MacLeod and Gordon, 1933; MacLeod, 1936). MacLeod (1936) found that the agent survived in infected ticks for long periods. I. ricinus can survive unfed for over one year awaiting a new host and it was interesting that the agent also

survived for over 14 months in its invertebrate host. This has a very significant epidemiological consequence. Ticks infected in their previous instar can still be infective after long periods of hibernation.

Although I. ricinus has been established as a definite vector of the disease, circumstantial evidence which implicate other ticks has been presented. Raghavachari and Reddy (1959) described an organism, Cytocoetes ovis var decanni as a cause of TBF in India. They thought that the disease was transmitted by the tick Rhipicephalus haematophysoides. Despite the mild nature of the disease described in India, there is little doubt that it is TBF (Tuomi, 1966).

MacLeod (1962) suggested that the reported presence of TBF in certain areas of England, the Somerset-Dorset border (Venn and Woodford, 1956) and the Winchester area (Tutt and Loving, 1955), indicated that there might be vectors other than I. ricinus. He argued that the occurrence of an immunological variant in cattle apparently more benign than the ovine strain, might be due as much to a change of vector, possibly Haemophysalis punctata, as to the change of vertebrate host, but he gave no evidence in support. The disease has also been reported from South Africa where I. ricinus is reportedly not present (Retief, Neitz and McFarlane, 1971). The transmission of the disease by subcutaneous or intravenous inoculation of TBF reinforced the possibility that other biting insects might also serve as mechanical vectors of the disease from one animal to another (MacLeod, 1932).

Animal Reservoirs

Sheep which have recovered from the disease harbour the organism in their peripheral blood for long periods. MacLeod (1932) found that blood of sheep which had experienced TBF was infective for 44 days. MacLeod and Gordon (1933) found that sheep which had recovered from clinical disease might act as reservoirs for some considerable time and they suggested such animals might serve as continuous sources of infection for ticks. Foggie (1951) found that the infective agent of TBF persisted in the blood. In one sheep, he found that the blood was infective for more than two years. In contrast, cattle appear to be poor carriers (Hudson, 1950; Tuomi, 1967[a]). The isolation of the organism from wild animals (Foggie, 1962[a]; McDiarmid, 1965; Greig, 1969) raises the possibility that they may act as natural reservoirs of the disease.

The survival of the organism in the invertebrate host for long periods (MacLeod, 1936), the presence of the organism in the blood of sheep months after they have recovered from clinical disease (Foggie, 1951) and the presence of wildlife reservoirs (Foggie, 1962) ensure the continued maintenance of the disease.

AETIOLOGY

Morphology

The nature of the causative agent of TBF was not known until Gordon and his colleagues (1940) reported that the organism occurred in the cytoplasms of the granular leucocytes and monocytes; a close study of the reports of the Moredun Institute, however, shows that "cytoplasmic inclusions" were found in the white cells of peripheral

blood and spleen as early as 1932 but the workers concerned hesitated to publish their findings for eight years (Anon., 1933; Anon., 1936). McEwen (1947) reported that the inclusion bodies stained with varying intensity by the Romanowsky stains and were pleomorphic in appearance. He described them as round, with diameters of 0.3μ to 0.5μ, or they were irregularly-shaped.

Hudson (1950) gave a detailed description of the various forms of the organism in the cytoplasm of the leucocytes of affected cattle. He found the causative agent in the granulocytes, metamyelocytes and monocytes during and for a few days after the febrile reaction. He speculated that the appearance of various forms suggested a multiplication cycle in the cells. He thought that the organisms resembled very closely to Rickettsia canis (Donatien and Lestoquard, 1935), R. bovis and R. ovina (Donatien and Lestoquard, 1936).

Foggie (1951) made a major contribution to the understanding of the morphology of the agent. He set out to investigate whether the various morphological types commonly seen represented stages of development as displayed by what are now known as Chlamydia. After injecting sheep with infected blood, he took blood samples every three hours from the 48th hour onwards and studied the morphological type of each sample. He found that the "initial bodies" were the first to be detected and the "fragmenting forms", "clusters" and "morulae" were detected later but he did not find any clear sequence of development; the "initial bodies" predominating throughout the period of parasitaemia. Despite Foggie's findings (1951), however, the names popularly attributed to groups of organisms with

a clear cycle of development such as the Chlamydia have been retained for the TBF organism. According to Gordon, Brownlee, Wilson and MacLeod (1962), for instance, the small "elementary bodies" with a diameter of 0.5μ are the infective forms; this form apparently develops to give large homogenous masses or "initial bodies" and the latter develop to "morulae" or fragment to form "clusters".

Ultrastructure

The first ultrastructural study of the organism in the leucocytes of infected sheep and cattle was carried out by Tuomi and von Bonsdorff (1966). This was an important contribution to the understanding of animal rickettsias in general and TBF in particular. They demonstrated that what were appearing as irregularly-shaped masses under the light microscope were aggregates of particles closely packed together inside cytoplasmic vacuoles. The vacuoles were separated from the host cell cytoplasm by a limiting membrane. The organisms inside the vacuole were surrounded by a rippled double-membrane and their arrangements inside the vacuoles were variable thus providing an explanation for the different forms seen under the light microscope. Having observed that there were particles of various sizes in the ovine strain and only large particles in the bovine strain, they suggested that there was a life cycle but did not provide any other evidence to support this hypothesis. In the invertebrate host ultrastructural studies were made by Lewis (1978). He reported that he detected the organism within the ovaries of female I. ricinus but did not elaborate

whether he also detected it in other parts of the tick and it is not clear whether the "rickettsia-like" organisms he observed were truly C. phagocytophila for it is common to find naturally occurring "rickettsia-like" symbionts in the ovaries of ticks (Weiss, 1974).

Physical and Chemical Properties

Plasma and serum of infected blood contain the organism (Foggie, 1951). Plasma and serum passed through 1.5 μ filters were infective but the organism was retained by 1.0 μ filters. The organism was reported to remain viable for up to ten days in citrated infected blood at room temperature (Foggie, 1951) and for 14 days at 4°C to 8°C (Hudson, 1950). Foggie, Lumsden and McNeillage (1966) found that the organism retained its infectivity for at least 18 months when small volumes of infected blood were kept at -79°C in the presence of glycerol or dimethyl suphoxide as cryoprotectant. Tuomi (1967[a]) reported that the agent kept its infectivity for up to 8 months at -74°C while Peirce, Norton and Donnelly (1974) found that the bovine strain of C. phagocytophila retained its infectivity at -196°C in liquid nitrogen with or without cryoprotectants for 11 months.

Sensitivity to Drugs

Sulphamezathine was believed to suppress the organism in the bloodstream but infection was not always eradicated (Foggie, 1951; Tuomi, 1967[e]). The organism is reportedly sensitive to oxytetracycline (Venn and Woodford, 1956; Overas, 1959; Foggie, 1951; Foggie and Allison, 1960; Tuomi, 1967[e]; Synge, 1976). It was

resistant to penicillin (Foggie and Allison, 1960; Tuomi, 1967[e]; Synge, 1976), chloramphenicol (Tuomi, 1967[e]; Evans, 1972; Synge, 1976), streptomycine and ampicillin (Synge, 1976). The organism was found also to be relatively sensitive to "Gloxazone" (alpha-ethoxyethylglyoxal dithiosemicarbazone) and to tylosin (Evans, 1972; Synge, 1976).

Classification

The classification of the organism has been beset with controversy and is full of contradictions. Early workers tried to classify the organism without any knowledge of its morphology and modes of multiplication. Gordon and his colleagues (1932), for instance, from the outset thought that the organism belonged to the Rickettsia. Further information on the nature of the organism has resulted in the continuous amendment of its tentative classification. Foggie (1951) suggested that the organism should be classified with the rickettsias, as Rickettsia phagocytophila, on the basis of its intracellular location, its arthropod vector and its morphological resemblance to what are now known as Chlamydia. However ten years later he rejected that classification, claiming that apart from an arthropod vector the organism of TBF had little resemblance to true rickettsias such as those causing typhus and Rocky-Mountain spotted fever (Foggie, 1962[a]). He also rejected any association with the Chlamydia because he did not find any serological or immunological connection between the TBF agent and the Chlamydia causing enzootic abortion in ewes (Foggie, 1962[b]). On the other hand, he argued that the organism had striking morphological similarities with Cytocoetes

microti, an organism which was found in the polymorphonuclear cells of the vole (Tyzzer, 1938). Foggie (1962[b]), therefore, proposed that the organism should be included in the genus Cytocoetes in the tribe Ehrlichiae (Moshkovskii, 1945) to distinguish it from the genus Ehrlichia of the same tribe ; the latter being reserved for those animal rickettsias which invaded mononuclear leucocytes.

Tuomi and von Bonsdorff (1966) reported that they had evidence based on ultrastructural studies which indicated that the organism should be placed in an intermediate position between the Rickettsia and Chlamydia. Their conclusion was based on their observation that in samples prepared from an ovine strain there were small, intermediate and big particles, indicating a life cycle and hence similarity to the Chlamydia. Their observation of only big particles in samples prepared from a bovine strain indicated no life cycle.

In the latest edition of Bergey's Manual of Determinative Bacteriology (Philip, 1974) the organism has been classified as Ehrlichia phagocytophila. However, the classification of the organism has not been definitely settled. While some authors regard the organism as belonging to the genus Ehrlichia (Philip, 1974; Purnell and Brocklesby, 1978; Greig, MacLeod and Allison, 1977; Thrusfield, Synge and Scott, 1978) others prefer to put it in the genus Cytocoetes (Tyzzer, 1938) as Cytocoetes phagocytophila (Evans, 1972; Retief et al., 1971; Snodgrass, 1974; Peirce et al., 1974; Synge, 1976; Lewis, 1979).

Cultivation

Attempts to cultivate the agent in bacteriological media have been unsuccessful (Gordon et al., 1932; Tuomi, 1967[a]). The organism

has not been propagated in embryonated hen eggs (Hudson, 1950; Tuomi, 1967[a]). Various cell culture systems have failed to support the growth of the organism *in vitro* (Tuomi, 1967[a]; Thrusfield *et al.*, 1978).

Strain Differences

Ever since Hudson (1950) reported TBF in cattle some workers have presented evidence indicating the existence of ovine and bovine strains of *C. phagocytophila*. The differentiation of strains was, however, based on ill-defined criteria. Hudson (1950), for instance, said that the bovine strain caused less severe infections in sheep than the ovine strain but did not elaborate how he measured severity in such a mild disease. Foggie (1951) reported that four Ayrshire heifers and one nine-month-old Ayrshire bullock and a 13-month-old Galloway heifer failed to react to an ovine strain but whether this was due to an innate resistance attributable to young animals (McEwen, 1947; Tuomi, 1967[a]) or was due to host specificity was not clarified by the inoculation of one cow which reacted mildly. Foggie and Allison (1960) could not regularly infect sheep with the bovine strain. They inoculated nine sheep with blood from infected cattle and only four reacted mildly. When two of those which reacted were challenged with an ovine strain, after an unspecified time, they reacted clinically. Tuomi (1967[b]) found that most bovine strains were less virulent to sheep while a few were as virulent to sheep as they were to cattle.

Differentiation among the so called ovine and bovine strains has been very difficult, the main problem being the measurement of virulence. Some workers said one strain was more virulent than

another without specifying what parameters they were using to assess virulence while others have used specified parameters for this purpose but found difficulty in quantifying them.

Foggie (1951) reported that strains of tick-borne fever varied greatly in virulence and that depending on the virulence of the infecting strain the temperature might drop rapidly or might show a more gradual fluctuating decline lasting ten or 12 days. Tuomi (1967[b]) used the percentage of infected granulocytes, the period of fever, the degree and durations of parasitaemia and the length of the incubation period to assess the virulence of bovine strains in Finland. He also used cross-immunity tests to differentiate them, but his challenge schedules were so inconsistent that it is difficult to interpret his results. In contrast, Foster and Cameron (1970[a]) could not differentiate two ovine strains on the basis of cross-immunity or virulence.

CLINICAL SIGNS

Incubation Period

Under natural conditions the incubation period varies in length. Gordon and his colleagues (1932), for instance, reported that it might vary from four to eight days. MacLeod and Gordon (1933) found that in experimental infections with ticks the incubation period was shorter after infestation with males than that after infestation with females. In the former case it was usually between four and six days but varied from three to seven days, but after infestations with females they found that the incubation period was about seven to eight days although it might vary from five to

13 days. Tuomi (1966) had little success with experimental transmission of the disease from ticks, but one cow reacted clinically 12 days after infestation with unfed nymphs collected from pasture.

There is enough evidence to suggest that the incubation period is considerably shorter and less variable when sheep are injected with infected blood but in infections affecting cattle it is widely variable.

Thus many workers (MacLeod, 1932; Gordon et al., 1932; MacLeod and Gordon, 1933; Foggie, 1951; Snodgrass, 1974) reported that the incubation period following intravenous inoculation of sheep with infective blood was usually three to four days. Whereas the incubation period in cattle was usually four to 11 days (Hudson, 1950; Tuomi, 1967[a]). It is not clear whether increased length was due to strain or host differences. Hudson (1950), for instance, reported that the incubation period in 12 cows infected with an ovine strain was four to 11 days, similar to 54 cases of cattle infected with a bovine strain. It has been reported that the incubation period might be lengthened as a result of storage of infected blood in certain environmental conditions (Hudson, 1950; Tuomi, 1967[a]).

Fever

The main clinical feature of the disease is fever. MacLeod and Gordon (1933) found that the disease started with an abrupt rise in rectal temperature. The highest point of the thermal reaction was usually on the second day. The duration of fever appears to be variable. Gordon and his colleagues (1932), for example, observed that febrile reactions lasted for about ten days

but other workers have reported short febrile periods. MacLeod and Gordon (1933) reported that in infections caused by tick infestations the febrile period was usually of about 12 days' duration but might vary from six to 22 days while in infections caused by inoculations of infected blood the febrile period was short.

Hudson (1950) said that 63 percent of 54 cattle had temperature reactions of three or four days. Foggie (1951), on the other hand, reported that depending on the virulence of the strain, the temperature might drop rapidly or may last ten to 12 days but he did not give any data to corroborate this. There is no clear evidence of the reported differences in the febrile period between ovine and bovine strains as claimed by some workers (Hudson, 1950; Tuomi, 1967[b]).

Tuomi (1967[b]) and Foster and Cameron (1970[a]) tried to assess strain differences by comparing the incubation period, the febrile period and rate of parasitaemia but failed to find any concrete differences to differentiate strains. The febrile period is usually monophasic but secondary thermal reactions occur commonly (Hudson, 1950; Foggie, 1951; Tuomi, 1967[a]; Snodgrass, 1974).

Other Signs

Other signs are either absent or very mild. McEwen (1947), for instance, reported that the disease generally caused a temperature reaction of one to five or six days' duration without any other constitutional disturbance. However, during the febrile period the animals appeared dull and lost weight (Gordon et al., 1932; Gordon, 1934; Gordon, Brownlee and Wilson, 1940). The rate

of respiration was reportedly increased (MacLeod, 1932; Hudson, 1950; Tuomi, 1967[a]) and there was anorexia (Venn and Woodford, 1956; Overas, 1962; Tuomi, 1967[a]; Ganda, 1977). Perhaps of more economic significance was the observation that dairy cattle affected by TBF suffered from reduced milk yields (Hudson, 1950; Tutt and Loving, 1955; Venn and Woodford, 1956; Foggie and Allison, 1960; Tuomi, 1967[a]). Coughing has also been occasionally reported but whether it is attributable to TBF per se is not clear (Hudson, 1950; Tuomi, 1967[a]). Foster, Foggie and Nisbet (1968) described a rare haemorrhagic syndrome in sheep infected with TBF but could not reproduce it consistently.

Mortality

Unless complicated by other infections, TBF seldom terminates fatally. Gordon and his colleagues (1932) reported that the disease had low mortality. MacLeod and Gordon (1933) found that out of 75 cases produced by inoculation of infective blood, only two deaths directly attributable to TBF occurred and out of 20 cases experimentally produced by infestation with ticks only one died of TBF. Gordon (1934) described the disease as being seldom fatal in sheep infected in clean premises. Most of the reported cases of high mortality were pregnant gimmers and ewes which aborted after being moved from tick-free to tick-infested pastures.

Jamieson (1947), however, reported high mortality rates in hoggs that were moved to tick-infested pastures. He divided seven-month-old hoggs into two groups of 120 and 80. The first group stayed on tick-infested pastures while the second group was wintered in tick-free areas. In April he placed both groups on

tick-infested pastures. In the group which wintered in tick-free pasture 24 percent died of TBF or TBF-suspected cases compared to six percent of those which were wintered on tick-infested pastures. Despite the fact that he did not have absolute evidence of the presence of TBF in each case, he concluded that the cause was TBF. Evidence of deaths in sheep following an abortion due to or as a sequel to TBF was provided by Jamieson (1950) and Littlejohn (1950) but they did not elaborate whether the deaths were due to TBF *per se* or were due to the bacterial complications which follow abortion.

Predisposition to Other Conditions

TBF has been implicated as a predisposing factor to many conditions but most of the accusations were often speculative with only fragmentary evidences being presented. It has been claimed that louping ill might be aggravated by the simultaneous presence of TBF (MacLeod and Gordon, 1933; Gordon *et al.*, 1962; Foggie, 1962[a]). TBF has been accused also of aggravating pasteurellosis, Johne's disease and pneumonia (Stewart, 1936; Hudson, 1950; Foggie, 1951). McEwen (1947) thought that lambs infected with TBF might be adversely affected by bad weather. Naerland (1956) observed that attacks of TBF acted as predisposing factors to enterotoxaemia and braxy, especially on lush, late season pastures. Grønstøl and Ulvand (1977) implicated it with predisposition to septicaemia as a result of activating latent infections with *Listeria monocytogenes* and Greig, MacLeod and Allison (1977) considered TBF to have played a major role in a complex disease syndrome involving mucosal disease and cobalt deficiency in a group of young calves reared in an upland

farm in south-west Scotland.

The most important and widely reported complications of TBF infections are staphylococcal pyaemia in lambs and abortion of pregnant ewes and cows.

Pyaemia. The connection of TBF with lamb pyaemia was suspected very early. Gordon (1934) had observed that it was not uncommon to find many crippled lambs and in many cases TBF was accompanied by infections with pyogenic organisms. Taylor and his colleagues (1941) set out to investigate whether the pyaemia that followed superficial abscess formation was related to TBF but failed to demonstrate that concurrent infection with TBF was responsible for the dissemination of staphylococci. Their failure might have been due to the fact that they used four-month-old lambs, an age at which pyaemia seldom occurs. Subcutaneous inoculation of staphylococci was later found to cause only localised abscesses (Foggie, 1957; Foster and Cameron, 1968[a]).

Abortion. Mainly circumstantial, but also some experimental, evidence has been presented connecting TBF with abortions. Stewart (1936) was first to suggest the disease as a cause of abortion and this was quickly confirmed by other workers. In a long review of the causes of abortion, Harbour (1945) cited TBF as a possible cause. During the period 1943-1944 he observed abortions of up to 30 percent of pregnant ewes when they were removed from tick-free to tick-infested pastures in Scotland. Harbour (1945) also observed that when sheep were acclimatized to tick-infested farms they aborted less.

In order to investigate Harbour's (1945) observations, Stamp and Watt (1950) experimentally inoculated ten pregnant Blackface gimmers susceptible to TBF and ten pregnant Blackface ewes born and reared in tick-infected pastures. All sheep in the first group developed TBF and five of them aborted and one was barren. None of the acclimatised sheep developed TBF or aborted; eight lambed normally, one was barren and one died from other causes. Jamieson (1950) presented field observations and experimental results confirming Stamp and Watt's (1950) findings. He found that all but one of ten susceptible pregnant ewes which were inoculated with TBF gave birth prematurely; all lambs were born dead or lived only a short time. Littlejohn (1950) similarly reported three outbreaks of abortion in sheep, usually gimmers, which were introduced into tick-infested pastures in south-east Scotland. She reproduced TBF in susceptible sheep with pooled blood from ewes which aborted. Overas (1959) reported abortions in ewes as a result of natural and experimental infections with TBF in Norway. It is not clear whether TBF causes abortions by itself or it only acts as a stress factor triggering abortions.

The only reported cases of cows aborting as a result of TBF were those described by Venn and Woodford (1956) and Wilson, Foggie and Carmichael (1964). The former reported infertility and early embryonic deaths during an outbreak of TBF in a herd of cattle in south-west England and the latter workers reported that from 120 in-calf heifers introduced into tick-infested pastures in north-west Scotland 28 aborted or produced dead calves within two months but they detected the organism in the blood of only five heifers which

aborted. Tuomi (1967[a]), on the other hand, did not find cases of abortion attributable to TBF which led him to suggest that Finnish strains of bovine TBF were less apt to cause abortion.

Two cases have been reported which implicate TBF with infertility of rams and bulls. Watson (1964) reported that four out of 17 rams became infertile shortly after introduction to tick-infested pastures. Pooled blood samples from the rams injected into a susceptible ram produced TBF and semen abnormalities. He suggested that the pyrexia which accompanied TBF might be responsible for the impairment of spermatogenesis. Retief and his colleagues (1971) experimentally infected two bulls with C. phagocytophila. One bull reacted with fever which lasted for eight days and the organism was detected in the blood while the other did not react clinically. Both showed high degrees of abnormal spermatozoal counts.

PATHOLOGY

Clinical Pathology

Very significant haematological changes accompany the disease (Taylor et al., 1941).

Leucocytes. In a comparative study of experimental infections of TBF, louping ill and staphylococci, Taylor and his colleagues (1941) found that sheep infected with TBF had leucopaenia. Their findings were later confirmed by other workers (Hudson, 1950; Foggie, 1956; Tuomi, 1967[a]). The drop in leucocytes coincided with the onset of the illness, reaching its minimum at the end of the thermal

reaction and then returning to normal levels gradually (Tuomi, 1967[a]).

Neutrophils. The disease is characterised by marked neutropaenia which is preceded by a slight increase in the number of neutrophils which occurs two to four days after infection (Taylor, et al., 1941). Then the neutropaenia progressively develops, becoming very marked at about ten days post-inoculation (Foggie, 1956).

Lymphocytes. Taylor and his colleagues (1941) also observed a decrease in lymphocytes over a short period beginning four days after inoculation. Tuomi (1967[a]) explained that the lymphocytopaenia preceded the neutropaenia, the lymphocytes rising to normal levels at the end of thermal reaction while the neutrophils continued to diminish in numbers.

Eosinophils. The eosinophils are also affected (Taylor et al., 1941). They disappeared from the blood a few days after infection. Tuomi (1967[a]) and Snodgrass (1974) later reported that the eosinophils were depressed for at least two weeks.

Monocytes. A rise in the number of monocytes allegedly follows neutropaenia but at what stage of infection monocytosis occurs is not established. Taylor and his colleagues (1941) wrote, "The monocytes were irregular, showing only temporary increases". Tuomi (1967[a]) found a slight absolute, but not relative rise in the number of monocytes at the end of clinical reaction which lasted for a few days. Snodgrass (1974) observed that monocyte counts

changed less consistently..

Thrombocytes. Foster and Cameron (1968[b]) found that there was an apparent diminution in the number of circulating thrombocytes. A marked decline in the platelets was approximately coincident with the onset of febrile reaction. The period of thrombocytopaenia was relatively short, the platelets returning to normal within seven to ten days. They attributed these changes to probable depression of bone marrow activity but considering the fact that thrombocytopaenia appears and subsides rapidly bone marrow depression is an unlikely cause. Unruh (1977) confirmed the above workers findings on the depression of blood platelet levels but his thrombocyte counts were not as markedly depressed. Foster and Cameron (1968[b]) postulated that the occasional haemorrhagic syndrome that was observed in outbreaks of TBF (Foster et al., 1968) might be due to thrombocytopaenia and other contributory factors. In contrast, Unruh (1977) found that the coagulation and bleeding time were not affected by the mild thrombocytopaenia that he observed.

Other haematological changes. Taylor and his colleagues (1941) observed that there was a small but progressive diminution of haemaglobin in sheep infected with TBF and suggested that this might be due to an inhibition of haemaglobin production caused by an inflammatory process and not due to destruction because there were no anaemic changes in blood. Unruh (1977) found an increase in fibrinogen levels of sheep infected with TBF but did not obtain any evidence of changes in in vivo haemostasis or significant

coagulation defects.

Other changes. Scott and Koske (1976) found a significant increase in free fatty acids one day after the onset of parasitaemia and it persisted for five days. They found no changes in the levels of total serum proteins, albumin, calcium chloride, creatinine, acid phosphatase, alanine aminotransferase, aspartic aminotransferase and sorbitol dehydrogenase.

Gross Pathology

Remarkably little information is available on the gross pathological changes that may accompany the disease. The only reported pathological change is that of splenic enlargement by Gordon and his colleagues (1932) and Hudson (1950).

Histopathology

Gordon and his colleagues (1932) found no histopathological changes on the spleen, the mesenteric lymph nodes and the central nervous system but Hudson (1950) observed that the lymphoid organs of infected animals were drained of lymphocytes and that there were cloudy swellings of the hepatic cells and the convoluted tubules of the kidney.

Tuomi and von Bonsdorff (1966) made ultrastructural studies of infected leucocytes. They found the organisms in cytoplasmic vacuoles without any apparent structural damage to the host cells.

IMMUNITY

The state of immunity of animals which had experienced an infection with TBF is not clear. Most of the information available

is speculative and often contradictory. Many workers have reported the existence of "absolute immunity", "relative immunity", "solid immunity", etc. but nowhere is it clear what immunity to TBF is. Some meant lack of thermal reaction. Others talked of premunition (Foggie, 1951) but evidences of sterile immunity have also been presented (Tuomi, 1967[a]).

All the available information indicates that there is some degree of protection against a serious manifestation of the disease upon re-exposure to the disease but the degree and duration of such immune state and the mechanism by which it is achieved are ill-defined.

Innate Resistance

There are some indications that some resistance to the disease based on age or breed may be present. Some workers thought that young lambs and calves are less susceptible to the disease (McEwen, 1947; Jamieson, 1947; Tuomi, 1967[a]), while others have pointed out that the disease might be more acute in lambs (Gordon, 1934). McEwen (1947) found that many lambs did not show any febrile reaction upon exposure to the disease but because he did not take blood films regularly he did not know whether they had parasitaemic reactions or not. Tuomi (1967[a]) found that even though calves were affected as early as the first week of life, the reactions of calves were less severe than those of adults.

The reaction in young animals might be mild, but the indications are that the organism invades the leucocytes of young animals as early as the first days of their lives (McEwen, 1947; Jamieson,

1947; Foggie, 1956, 1957; Foster and Cameron, 1968[a]; Tuomi, 1967[a]). Scott and Koske (1976) confirmed that young lambs reacted less severely than adults but they also found that aged adult sheep possessed similar high innate resistance.

Breed differences in natural resistance have been investigated by Tuomi (1966) and Scott and Koske (1976). The former found that incidences of TBF in the Juva region of Finland were more common in cattle brought from other regions than in indigenous ones but he did not elaborate whether this was due to acquired immunity in the indigenous cattle as a result of continued exposure to TBF or due to innate resistance. The latter authors found that Blackface sheep were less susceptible to TBF than other breeds and their crosses.

Acquired Immunity

That animals acquire some degree of resistance to reinfection after being exposed to the disease is undoubted. What is not clear is when this immunity develops and for how long it lasts.

Duration. Few workers have attempted to find how long after infection animals acquire immunity. Foggie (1951) claimed that resistance to re-infection develops five weeks after natural or experimental infections. He inoculated nine sheep with C. phagocytophila and then re-inoculated one sheep every week. Sheep re-inoculated after one, two, three, four and eight weeks reacted while those re-inoculated after five, six, seven and nine weeks did not react clinically. This led him to conclude that resistance to re-infection develops after five weeks but he did not establish

whether the reactions in the first few weeks were not due the secondary reactions that might follow primary infections (Hudson, 1950; Foggie, 1951; Tuomi, 1967[a]). Hudson (1950) reported that cattle infected with the bovine strain did not resist re-infection within a few days after the end of the first reaction, but if the presence of antibodies can be taken as a measure of immunity it seems that it develops within three weeks post-infection (Tuomi, 1967[d]; Snodgrass and Ramachandran, 1971).

Once animals have acquired resistance against re-infection, for how long does this resistance last? While some workers have claimed that it lasts for long periods, others have said it is short-lived. Stamp and Watt (1950) claimed, for instance, that one inoculation of 10 ml of infected blood was sufficient to confer a considerable degree of immunity to a similar challenge dose for a period of 12 months but a close scrutiny of their data reveals that out of six pairs of ewes, one pair of which was being challenged every two months, one sheep reacted after two months and three sheep reacted after five to six months. In contrast, Hudson (1950) said, "the failure of one attack to give complete immunity has made it difficult to design cross-immunity tests". Overas (1962) found that if infected sheep were removed from tick-infested pastures for a period of three and a half months they developed TBF upon re-entry to the pastures. Tuomi (1967[a]) attempted to study the duration of protective immunity in cattle infected with TBF but his experimental designs were such that it is difficult to draw any conclusions from them. For example, he claimed that most of 30 cases challenged within three and a half

months were immune but a study of his immunization schedule shows that most of them were challenged within three weeks of primary reaction. He claimed that two of five sheep challenged within five and three quarters and eleven and a half months reacted, but one of them was splenectomized before challenge.

Snodgrass (1974) reported that when 12 sheep were challenged ten months after primary reaction, none of them reacted clinically, but it is not clear whether they reacted with parasitaemia or not because he took blood samples only when animals had showed a thermal reaction above 40.5°C.

Scott (1975) found that 80 percent of sheep challenged within six months of primary infection, 53 percent of those challenged after six months and 32 percent of those challenged after 64 weeks were immune to homologous challenge.

Some workers have claimed that continuous exposure to the disease is necessary for immunity to last long while others have found no evidence to support that. Gordon and his colleagues (1932) observed that sheep resisted re-infection after one or several inoculations. Harbour (1945) suggested that sheep which were continuously exposed to TBF did not abort because they developed immunity to TBF. Jamieson (1947) emphasised that pregnant ewes from tick-free farms introduced to tick-infested pastures aborted more frequently in contrast to those kept continuously in tick-infested farms. If hoggs which were reared in tick-infested farms were removed to tick-free farms for six months they developed TBF when they were re-introduced to tick-infested pastures compared to those which stayed in the tick-infested pastures which did not

develop TBF. Overas (1962) reported similar findings. Scott (1975), on the other hand, found that frequent exposure to TBF did not necessarily enhance the duration of immunity.

Cross-immunity

After Hudson (1950) described the disease in cattle some workers investigated the possibility of cross-protection between the ovine and bovine strains but they got conflicting results, mainly because measurement of immunity was not properly defined. Hudson (1950), for example, claimed that there was a considerable cross-protection between ovine and bovine strains but his experimental results do not seem to warrant such a conclusion. He found that most cattle which were previously infected with either the bovine or ovine strain resisted reinfection by a bovine or ovine challenge. In contrast, all the sheep which were previously infected with a bovine strain reacted when challenged with an ovine strain.

Foggie and Allison (1960) claimed that there was no evidence of immunity to ovine strains following infections by bovine strains. They experimentally infected two sheep with an ovine strain and two sheep with a bovine strain and then challenged them with an ovine strain. Those which were infected previously with the bovine strain reacted clinically while those which were infected with an ovine strain did not. Tuomi (1967[c]) found that Finnish bovine strains did not confer protection against Scottish ovine strains and vice versa.

Cross-immunity trials were carried out with ovine strains by Foggie (1951) and Foster and Cameron (1970[a]) and with bovine strains by Tuomi (1967[b, c]). Foggie (1951) infected four groups of eight, eight, nine and nine sheep with four different ovine strains. Five weeks later he challenged two or three animals from each group with a homologous strain or with one of the heterologous strains. He found that with homologous challenge two animals reacted and six did not while with heterologous challenge 17 sheep reacted and nine did not. He subjected his results to statistical analysis and concluded that there was a statistically significant difference between the number of animals which reacted to homologous challenge and the number of those which reacted to heterologous challenge, the Chi-square value being 4.047. The number of animals he used, however, was small and Yate's correction should have been applied; the corrected Chi-square value is 2.575 which is not statistically significant. A close study of his tabulated data revealed that some of the strains were similar, if not identical. Animals infected with strains B and C, for example, responded to homologous and heterologous challenge in a similar way and there was no significant difference between strains B and F. More animals reacted to heterologous challenge, but this is because fewer animals were used for homologous challenge.

Tuomi (1967[c]) undertook cross-immunity trials among 11 Finnish bovine strains and reported that it was rather exceptional for infection by one strain to result in the protection against infection by another strain. However, his immunization and challenge schedules were haphazard and inconsistent. For instance, he did not clearly

spell out when the challenge inoculations were carried out. He said, "usually serial inoculations were made within three weeks after subsidence of the reaction induced by previous strain or strains; on occasion the interval was longer, although a few inoculations were made at the time when the preceding reaction was still in progress".

Foster and Cameron (1970[a]) found that two ovine strains isolated from the Southern Uplands of Scotland conferred protection against each other, indicating immunological identity. However they were taking blood samples after the third, the sixth and ninth day after challenge only and challenge inoculations with a heterologous strain were carried out 12 days after homologous challenge. Thus they might have missed reactions that were accompanied by a parasitaemia of short duration (Scott, 1978).

Mechanisms of Immunity

The mechanism by which animals which had experienced a TBF infection develop immunity is ill-defined. Some workers have speculated that it might be due to a state of premunition or carrier state while some degree of sterile immunity has not been ruled out (Hudson, 1950; Foggie, 1951; Scott, 1978).

Carrier state. It has been recognized for a long time that the organism remains in the blood of sheep which had recovered from clinical disease for long periods (Foggie, 1951). This has led some workers to postulate that persistence of the organism might be necessary for the maintenance of an immune state against the serious manifestation of the disease upon re-exposure (Foggie, 1951).

However, the carrier state does not seem to be absolutely necessary for an immune state. Hudson (1950), for example, contended that there could be a period of premunition followed by a period of sterile immunity during which the blood of immune animals did not contain infective organisms but the animals resisted re-infection. Foggie (1951) challenged a sheep whose blood was not infective 32 months after infection without causing clinical reaction. Tuomi (1967) could only isolate the organism from blood of infected animals for a few days and yet he reported that most animals resisted re-infection at least for the first few months.

Scott (1978) found that carrier sheep always resisted re-infection and that the proportion of sheep immune to re-infection fell steadily after the first weeks of convalescence and the rate of decline of immune animals parallelled that of carrier sheep, but the number of immune sheep was higher than the number of carriers. He found that 12 months after infection 70 percent of sheep which were not carriers were not reinfected upon challenge.

Humoral antibodies. McEwen (1947) observed that young lambs were more resistant to infection than older animals and he speculated that this might be due to antibodies they obtained through the colostrum of immune ewes. Tuomi (1967[d]) reported that he demonstrated immunofluorescent antibodies against bovine and ovine strains of TBF. Snodgrass and Ramachandran (1971) detected complement fixing antibodies in sheep two weeks after infection with ovine strains of TBF.

CHAPTER TWO

CLINICAL PARAMETERS

INTRODUCTION

The predominant clinical sign of sheep infected with TBF is fever (Gordon et al., 1932). Other clinical signs are either absent or are very mild (McEwen, 1947). However, the disease is accompanied with severe haematological disorders (Taylor et al., 1941) and parasitaemia (Foggie, 1951). The purpose of the present study is to record and analyse the febrile reaction and the changes in the leucocytes of sheep experimentally infected with C. phagocytophila to establish base-line data against which re-infections could be assessed.

MATERIALS AND METHODS

Cytocoetes phagocytophila

The Old Sourhope (OS) strain of C. phagocytophila was used. This strain was first isolated by Foster and Cameron (1970[a]) from sheep on farm S in the county of Selkirkshire in the southern uplands of Scotland. Stabilates of the organism were prepared according to the method described by Foggie and his colleagues (1966), with some modifications. Briefly, blood was collected from infected sheep at the peak period of parasitaemia in tubes containing heparin to give a final concentration of 20 I.U. of heparin per ml of blood. Dimethyl sulphoxide (DMSO) was added to the blood to make a final concentration of ten percent. The DMSO-treated infected blood was thoroughly mixed and distributed in aliquots of two ml in screw-capped glass bottles and stored in the vapour phase of a liquid-nitrogen refrigerator at -114°C.

Before use the infective blood was thawed quickly under running water and diluted ten-fold with phosphate-buffered saline (PBS) at pH 7.2. The inoculum was kept in an ice bath until use.

Animals

Adult sheep of mixed breeds, ages and sexes were obtained from tick-free areas. A total of 18 sheep were inoculated with one ml of a 10^{-1} dilution of the cryopreserved stabilate of C. phagocytophila intravenously.

Observations

Rectal temperatures and blood samples in dipotassium ethylenediamine tetra acetic acid (EDTA) were taken daily. Blood smears were prepared and stained with Giemsa or acridine orange stains and examined under oil immersion with a Nikon microscope for the presence of cytoplasmic inclusions in the granulocytes and monocytes (Gordon et al., 1940; Foggie, 1951). The presence of typical inclusions was taken as an indication of infection. At least 200 leucocytes were examined before declaring a sample negative.

For quantitative estimation of cells and parasitaemia, total leucocyte counts were estimated electronically (Coulter Electronics Ltd.) and used to calculate absolute numbers of neutrophils, eosinophils, lymphocytes and monocytes. Total infected cells, total infected neutrophils and total infected monocytes were similarly calculated.

The duration of fever was the number of days in which the rectal temperature was above 40.5°C. The magnitude of fever for the whole period was estimated from plots of daily temperature on

on five mm grids and calculated according to the Trapezium Rule (Hale, 1958). For this purpose 40.6°C was taken as a base line (Figure 1).

The duration of parasitaemia was the number of days on which organisms were detected. The magnitude of parasitaemia for a particular sheep throughout the period of parasitaemia was expressed in terms of the areas bounded by the graph of daily parasitaemias plotted on five mm grids and calculated according to the Trapezium Rule (Figure 2).

Analysis of Data

Means and standard deviations for all the parameters, except for the monocytes and eosinophils, were calculated according to conventional methods. The latter were not normally distributed and, therefore, their medians were calculated.

RESULTS

Rectal Temperature

All sheep which were inoculated with C. phagocytophila reacted with fever. The mean incubation period from inoculation to above 40.5°C was 3.18 ± 0.88 days. The clinical disease was characterised by a sudden rise in rectal temperature (Figure 1 and Table 1). Peak thermal reactions were recorded 2.24 ± 1.39 days after initial reaction (Table 2). The febrile period lasted for 6.47 ± 2.94 days and the total magnitude of fever was 2201 ± 942 mm^2 (Table 2). Some animals showed many peaks of febrile reaction while others had only monophasic reactions.

Haematological Changes

Severe haematological changes affecting the total leucocytes, lymphocytes, neutrophils, eosinophils and monocytes were observed.

Total leucocytes. The total leucocytes decreased from $9.4 \times 10^9 \pm 3.4 \times 10^9$ cells per litre before inoculation to a nadir of $4.6 \times 10^9 \pm 1.8 \times 10^9$ cells per litre on the seventh day post-inoculation. The total leucocytes remained at low levels for two weeks (Figure 3 and Table 3).

Lymphocytes. The lymphocytes were reduced from a mean of $5.9 \times 10^9 \pm 2.1 \times 10^9$ cells per litre before inoculation to a nadir of $2.8 \times 10^9 \pm 1.6 \times 10^9$ cells per litre on the seventh day post-inoculation (Figure 3 and Table 3). The nadir of lymphocytopaenia occurred after 5.67 ± 1.75 days post-inoculation. They remained at low levels for about 14 days post-inoculation, returning to pre-inoculation levels thereafter.

Neutrophils. The total neutrophils were increased from a mean pre-inoculation level of $2.4 \times 10^9 \pm 1.3 \times 10^9$ cells per litre to $4.3 \times 10^9 \pm 2.3 \times 10^9$ per litre on the second day post-inoculation. The neutrophilia, however, was transient. A marked and persistent reduction of total neutrophils followed, becoming very severe nine to eleven days post-inoculation. The nadir of $0.8 \times 10^9 \pm 0.3 \times 10^9$ cells per litre was reached on the eleventh day post-inoculation (Figure 3 and Table 3). The nadir of neutropaenia occurred after 11.00 ± 1.71 days post-inoculation, considerably later than that of lymphocytopaenia (Table 2).

Eosinophils. The eosinophils literally disappeared from the peripheral blood after the fourth day post-inoculation and did not return to pre-inoculation levels after 21 days post-inoculation (Figure 3 and Table 4).

Monocytes. The monocytes started to increase in numbers after the fifth day post-inoculation and remained at high levels for up to the eleventh day post-inoculation (Figure 3 and Table 4).

Parasitaemia

All the sheep inoculated with C. phagocytophila reacted with parasitaemia. The mean prepatent period from inoculation to first day of detection of the organism in peripheral blood was 3.18 ± 0.39 days. The first day of parasitaemia usually coincided with the first day of fever; it preceded the febrile reaction by one day in four sheep while the thermal reaction occurred before parasitaemia in six sheep. Peak parasitaemia of $2.01 \times 10^9 \pm 0.98 \times 10^9$ infected cells per litre was recorded 2.06 ± 0.87 days after initial detection of the organism (Tables 1 and 2). The organism was detected in the peripheral blood for a mean period of 7.3 ± 1.0 days. The mean magnitude of parasitaemia was 4899 ± 644 mm^2. The cells which were predominantly infected were the neutrophils but the organism was also detected in the eosinophils and monocytes. The latter were usually infected at later stages of the disease. Studies on a group of seven sheep showed that the monocytes were not infected until the seventh day post-inoculation; the monocytes being infected during the last one or two days of parasitaemia (Table 5). Some sheep showed monophasic parasitaemia while others had two or more peaks of parasitaemia.

Figure 1 Magnitude of fever (Sheep No. 120)

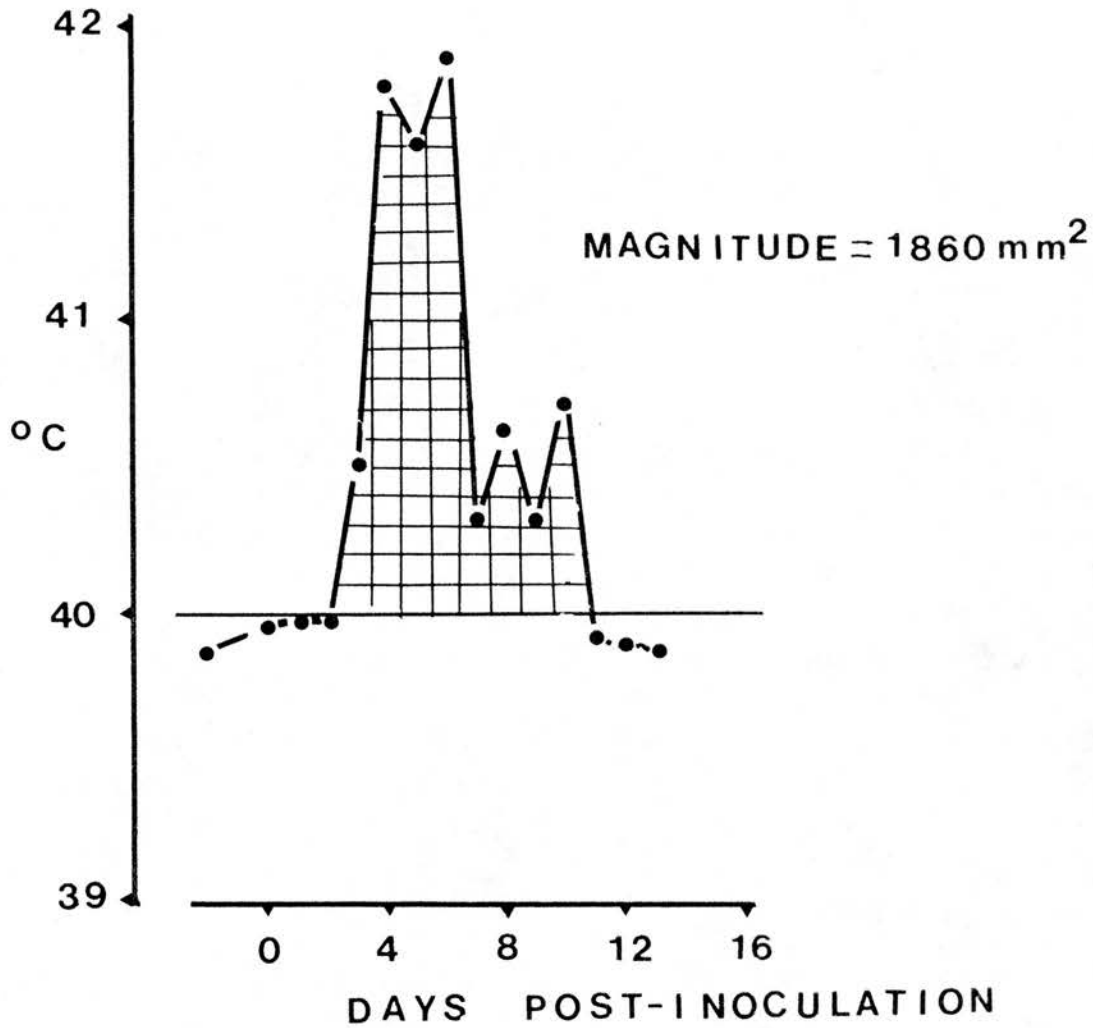

42
41
40
39
°C
MAGNITUDE = 1860 mm²
0
4
8
12
16
DAYS POST-INOCULATION

Figure 2 Magnitude of parasitaemia (Sheep No. 120)

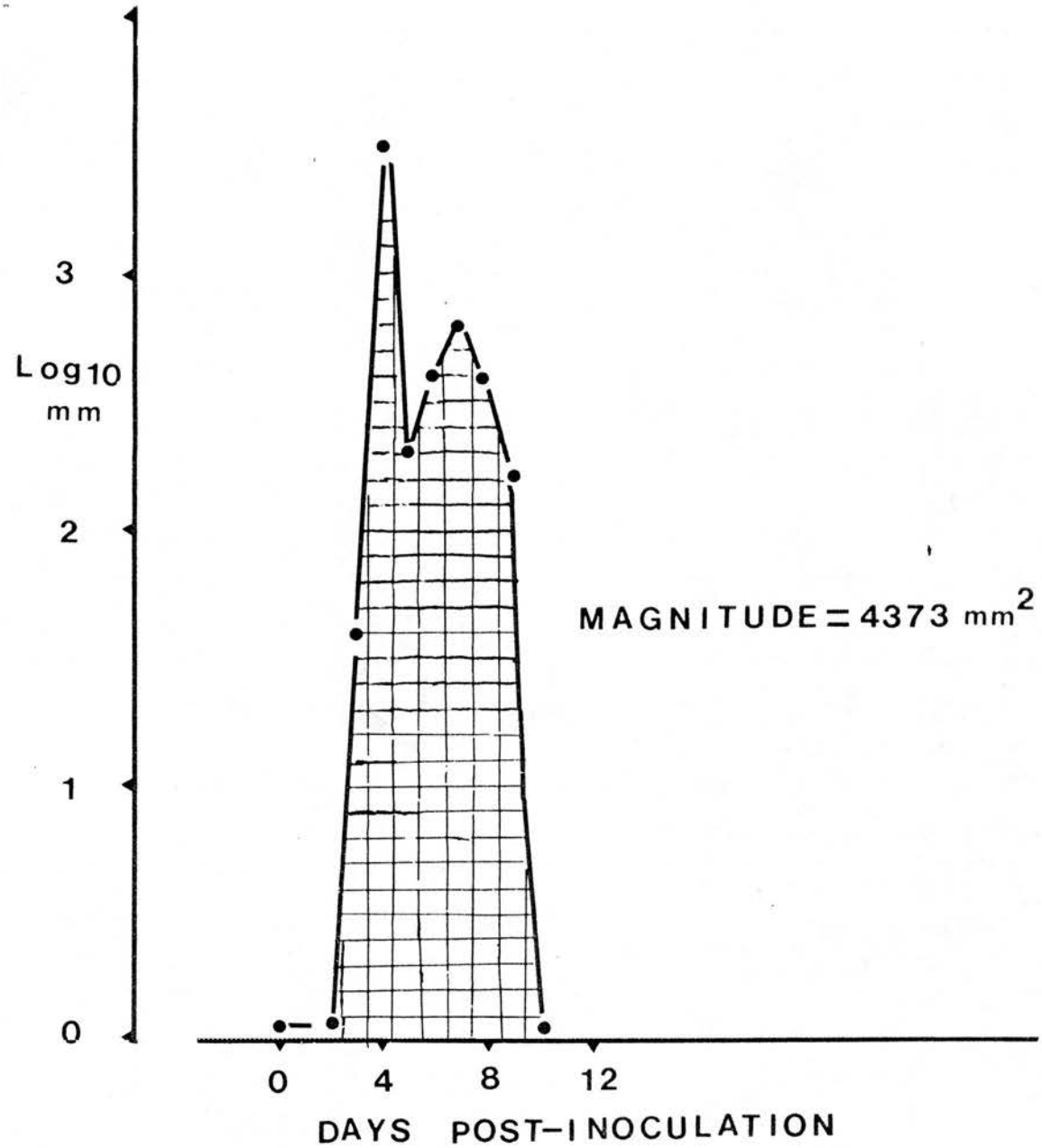

3
Log10
mm
2
1
0
MAGNITUDE = 4373 mm2
0
4
8
12
DAYS POST-INOCULATION

Figure 3 Mean total leucocytes of sheep infected with *C. phagocytophila*

A - total leucocytes

B - total lymphocytes

C - total neutrophils

D - total eosinophils

E - total monocytes

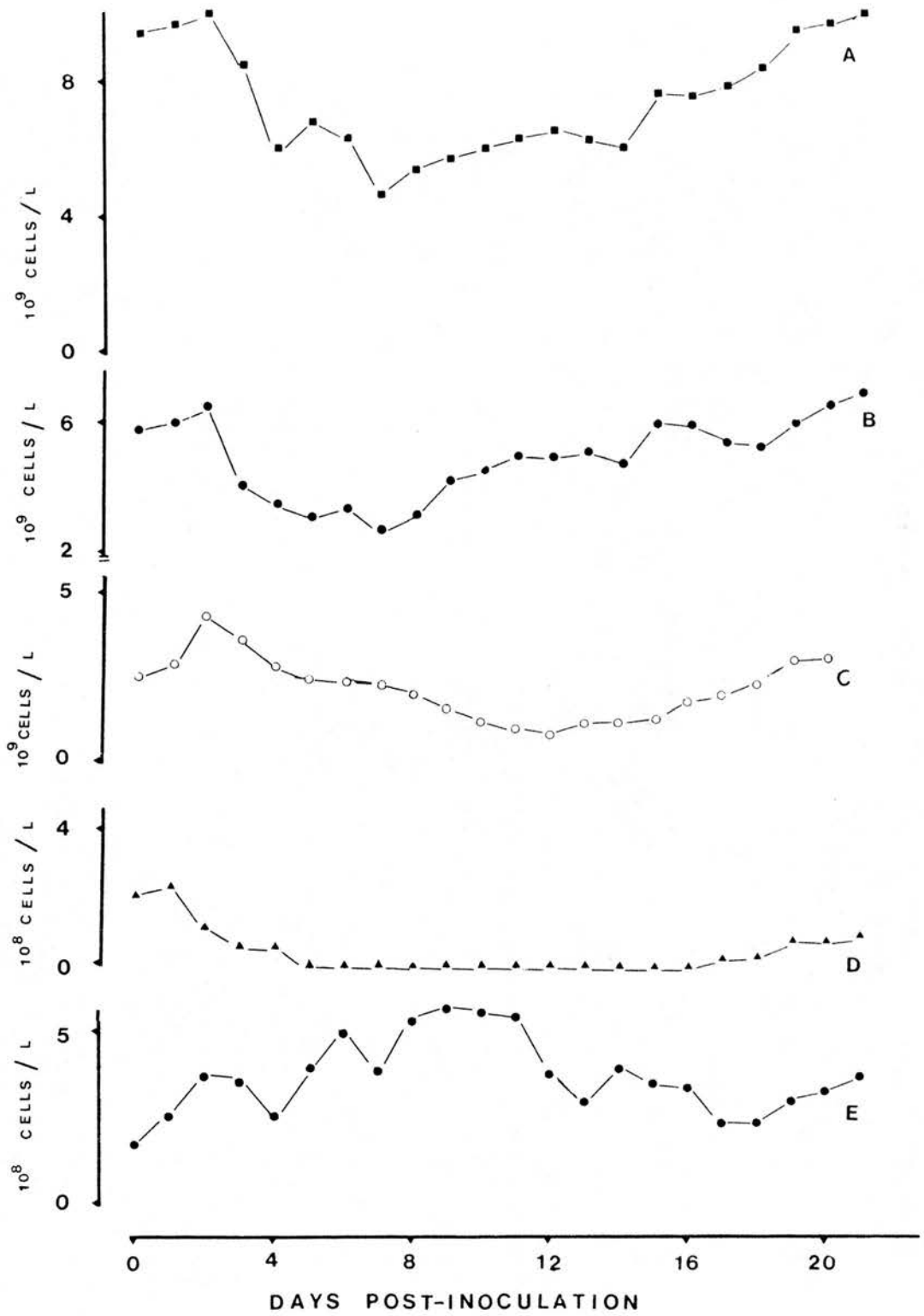

A
B
C
D
E
8
4
0
6
2
5
0
4
0
5
0
10^9 CELLS / L
10^8 CELLS / L
0
4
8
12
16
20
DAYS POST-INOCULATION

Table 1 Number of observations (n), mean temperature (°C) and parasitized cells ($x10^9$/litre)

Days post-inoculation	n.	Temperature Mean	Parasitized cells n.	Mean
0	18	39.7 ± 0.5[a]	15	0
1	17	40.0 ± 0.4	15	0
2	17	40.0 ± 0.6	11	0
3	17	40.9 ± 1.2	17	0.94 ± 1.02
4	17	41.3 ± 0.8	17	1.77 ± 1.03
5	17	41.4 ± 0.6	17	1.24 ± 0.96
6	17	41.4 ± 0.6	17	0.83 ± 0.66
7	17	40.8 ± 0.7	17	0.61 ± 0.47
8	17	40.7 ± 0.8	17	0.32 ± 0.22
9	17	40.3 ± 0.6	17	0.18 ± 0.17
10	15	40.1 ± 0.9	17	0.09 ± 0.13
11	17	39.6 ± 0.8	17	0.03 ± 0.08
12	17	39.7 ± 0.6	17	0
13	17	39.7 ± 0.6	17	0
14	16	39.8 ± 0.9	16	0

[a] standard deviation

Table 2 Number of observations, mean and standard deviations of clinical parameters of TBF

Parameter	Number of observations	Mean	Standard deviation
Prepatent period (to parasitaemia)	18	3.18	0.39
Peak (10^9/l) parasitaemia	18	2.01	0.98
Day of peak parasitaemia	18	2.06	0.87
Magnitude of parasitaemia (mm^2)	18	4899	644
Period of parasitaemia (days)	18	7.28	1.02
Incubation periods (to 40.5°C)	17	3.18	0.88
Peak fever (°C)	17	42.08	0.26
Day of peak fever	17	2.24	1.39
Magnitude of fever (mm^2)	17	2201	942
Febrile period (days)	17	6.47	2.94
Day (p.i.) of nadir of lymphocytopaenia	18	5.67	1.75
Day (p.i.) of nadir of neutropaenia	18	11.00	1.71

p.i. = post-inoculation

Table 3 Number of observations (n) and means (10^9 cells/l) of total leucocytes, neutrophils and lymphocytes

Days post-inoculation	Leucocytes n	Leucocytes Mean	Lymphocytes n	Lymphocytes Mean	Neutrophils n	Neutrophils Mean
0	17	9.4 ± 3.4[a]	15	5.9 ± 2.1[a]	15	2.4 ± 1.3[a]
1	16	9.7 ± 2.8	15	6.2 ± 2.0	15	2.8 ± 1.2
2	16	9.9 ± 3.8	10	6.5 ± 2.0	10	4.3 ± 2.1
3	17	8.5 ± 3.4	17	4.1 ± 2.0	17	3.5 ± 1.9
4	17	6.0 ± 2.6	17	3.7 ± 3.1	17	3.1 ± 1.5
5	17	6.8 ± 3.5	14	3.3 ± 1.9	14	2.8 ± 1.3
6	16	6.4 ± 2.8	14	3.5 ± 1.8	14	2.4 ± 1.1
7	17	4.6 ± 1.8	14	2.8 ± 1.6	14	1.8 ± 0.7
8	16	5.4 ± 2.2	14	3.3 ± 1.5	14	1.4 ± 0.7
9	17	5.9 ± 2.9	14	4.2 ± 2.4	14	0.9 ± 0.6
10	17	6.2 ± 2.8	14	4.6 ± 2.4	14	0.8 ± 0.4
11	17	6.4 ± 2.4	14	5.1 ± 2.0	14	0.8 ± 0.3
12	17	6.6 ± 2.0	14	5.0 ± 1.6	14	0.9 ± 0.5
13	17	6.3 ± 2.6	14	5.2 ± 2.0	14	0.9 ± 0.5
14	16	6.0 ± 2.9	15	4.8 ± 2.0	15	1.1 ± 0.8
15	9	7.9 ± 1.8	9	6.1 ± 1.7	9	1.5 ± 0.8
16	9	7.7 ± 1.5	9	5.8 ± 1.4	9	1.5 ± 0.8
17	9	7.9 ± 1.9	8	5.2 ± 1.8	8	1.7 ± 0.5
18	9	8.4 ± 2.3	8	5.2 ± 1.3	8	2.4 ± 1.0
19	9	9.7 ± 2.4	8	6.0 ± 1.8	8	2.5 ± 1.2
20	9	9.8 ± 2.3	8	6.5 ± 1.9	8	3.1 ± 1.7
21	8	10.4 ± 1.7	8	6.9 ± 2.0	8	3.1 ± 1.0

Table 4 Number of observations (n) and median of eosinophils and monocytes (x 10^9 cells/l)

Days post-inoculation	Eosinophils		Monocytes	
	n	median	n	median
0	15	0.20	15	0.16
1	15	0.22	12	0.25
2	10	0.11	10	0.37
3	17	0.05	17	0.35
4	17	0.05	17	0.25
5	17	0	17	0.40
6	17	0	17	0.50
7	17	0	17	0.38
8	17	0	17	0.54
9	17	0	17	0.58
10	17	0	17	0.56
11	17	0	17	0.55
12	17	0	17	0.38
13	17	0	17	0.31
14	16	0	16	0.40
15	9	0	9	0.36
16	8	0	8	0.35
17	8	0.03	8	0.24
18	8	0.04	8	0.24
19	8	0.08	8	0.31
20	8	0.08	8	0.33
21	8	0.10	8	0.38

Table 5 Number of observations (n) and mean parasitized neutrophils and monocytes (x 10^8 infected cells/l)

Days post-inoculation	Infected neutrophils n	Mean	Infected monocytes n	Mean
0	7	none	7	none
1	7	none	7	none
2	7	none	7	none
3	7	3.05 ± 2.76	7	none
4	7	10.78 ± 2.70	7	none
5	7	10.88 ± 8.02	7	none
6	7	7.25 ± 4.00	7	none
7	7	5.47 ± 3.10	7	none
8	7	3.90 ± 2.4	7	0.97 ± 0.25
9	7	2.5 ± 1.3	7	0.27 ± 0.40
10	7	1.2 ± 1.7	7	0.36 ± 0.50
11	7	0.10 ± 0.3	7	0.33 ± 0.60
12	7	none	7	0.04 ± 0.10
13	7	none	7	none
14	7	none	7	none

DISCUSSION

The data in this study confirmed other workers' findings about the prepatent period, the febrile reaction, parasitaemia and the haematological disorders that accompany TBF. MacLeod and Gordon (1933) had observed that the incubation period following inoculation of infected blood was three to four days, considerably shorter than the incubation period of infections due to infestations by ticks. In the present study the incubation period was also found to be short. The febrile reaction was characterised by a sudden peak lasting for about four days and then a gradual decline which lasted for a further four days. Most sheep had only one peak but others showed one or more secondary peaks at various intervals after the first phase. Measuring the magnitude of fever takes into account the various peaks. It is quantifiable and estimates the degree of thermal reaction throughout the period of clinical reaction. Similar estimations of the magnitudes of parasitaemia gave a better assessment of the degree of parasitaemia. The magnitudes of fever and parasitaemia may, therefore, serve as reliable parameters for comparing reactions due to different strains and comparing primary and secondary reactions.

The thermal reaction usually coincided with the first detection of the organism in the peripheral blood, but in some animals it either preceded paraistaemia by one day or it followed parasitaemia after one day. Parasitaemia was characterised by typical exponential growth, a peak and gradual decline (Snodgrass, 1974). The peak

period of parasitaemia was reached at the second day. This coincided with the period of neutrophilia. The percentage of infected neutrophils was higher at latter stages of infection but the reduction of total neutrophils reduced the absolute number of infected cells. Some workers have measured parasitaemia by percentage of infected granulocytes (Tuomi, 1967[a]). Such a measurement while showing relative increases in the number of infected neutrophils does not show the decreases in absolute parasitaemia at latter stages. The total infected cells per litre and magnitude of parasitaemia are, therefore, more accurate measurements of parasitaemia.

Monocytes were infected at latter stages of parasitaemia. This is similar to other workers' findings in bovine strains of C. phagocytophila (Tuomi, 1967[a]; Purnell and Brocklesby, 1978).

The haematological findings in this study agree generally with the findings of other workers. The lymphocytopaenia preceded neutropaenia. This is similar to the findings of Taylor and his colleagues (1941) and Tuomi (1967[a]). The eosinophils were affected slightly earlier than the neutrophils and in most animals they were absent from the peripheral blood for the whole period of the experiments. The monocytosis coincided with the time of infection of mononuclear cells. A slight increase in the number of monocytes of cattle infected with TBF was previously reported by (Tuomi (1967[a]) and monocytosis is a feature of the latter stages of canine ehrlichiosis (Ewing, 1969).

CHAPTER THREE

CULTIVATION OF *CYTOECETES PHAGOCYTOPHILA*

INTRODUCTION

Cytoecetes phagocytophila is readily transmitted by inoculating susceptible sheep and cattle with infected blood (MacLeod, 1932). Attempts to transmit the disease to laboratory animals have had only limited success (Foggie and Hodd, 1961) and the organism has not, hitherto, been propagated in embryonated hen eggs or cell culture systems (Thrusfield *et al.*, 1978). The present study was undertaken to explore new ways of propagating the organism *in ovo* or *in vitro*.

MATERIALS AND METHODS

Cytoecetes phagocytophila

The Old Sourhope strain (Foster and Cameron, 1970[a]) was used and handled as described in Chapter Two.

Infectivity of inoculum. To ensure that the inoculum used was infective it was important to demonstrate that the viability of the organism, preferably cell-free, was not affected by the conditions of culture. It has been reported that plasma and serum of infected sheep are infective (Foggie, 1951). The following preliminary experiments were, therefore, carried out to test the infectivity of plasma, serum and disrupted infected cells: plasma and serum were collected from infected sheep at the peak period of parasitaemia. They were filtered through 1.2μ and 1.5μ millipore filters and inoculated intravenously into susceptible sheep either immediately or after two hours at 37°C or 24 hours at 4°C. Infected leucocytes were disrupted by ultrasonic vibration at

optimum amplitude for two minutes and inoculated into susceptible sheep immediately or after 24 hours at 4°C. Serum and plasma inoculated into susceptible sheep immediately or after two hours at 37°C were infective but they were not infective after 24 hours at 4°C. In contrast, disrupted leucocytes were infective even after 24 hours at 4°C.

Antibiotics. Cytoecetes phagocytophila is reported to be sensitive to tetracyclines and sulphonamides (Tuomi, 1967[e]; Synge, 1976) but it is not sensitive to penicillin (Synge, 1976). The following preliminary tests were, therefore, carried out to test the sensitivity of the organism to penicillin and fungizone in vitro: infected cells were disrupted by ultrasonic vibration at optimum amplitude for two minutes and 1000 IU of penicillin and 50 µg of fungizone added to two ml of disrupted cell suspension. The mixture was left overnight at 4°C and two susceptible sheep inoculated with one ml of cell suspension intravenously. Both sheep reacted with TBF.

Culture Media

Commercially supplied medium 199 with or without HEPES buffer, minimum essential medium (MEM) and Roswell Park Memorial Institute 1640 medium (RPMI 1640) with HEPES buffer were used.

Serum supplements. Lamb serum (LS), foetal calf serum (FCS) and newborn calf serum (NBCS) were obtained from Gibco Bio-Cult (Glasgow). Autologous serum was obtained from animals whose leucocytes were used for macrophage cultures. The autologous sera were sterilised by millipore filtration. Cells were grown with 10

to 20 percent serum and maintained with five to 10 percent serum.

Some media were supplemented with 100 IU of penicillin and five micrograms of fungizone per ml immediately before use while others were used without antibiotics.

Egg Inoculations

The yolk sac of nine-day-old embryonated hen eggs were injected with 0.25 ml of infected blood or plasma. The eggs were kept at 37°C for five to ten days. Yolk sacs were harvested every 24 hours from at least two embryonated eggs at a time, from the third day onwards. Fat contaminants were reduced by centrifugation after disrupting yolk sacs by glass beads. The supernatant was discarded and the yolk sac material resuspended in sterile phosphate buffered saline (PBS) at pH 7.2. Smears were made and stored at -20°C until required. Blind passages were carried out by injecting yolk sac suspensions to embryonated eggs and on occasions suspensions of yolk sac material were inoculated subcutaneously into susceptible sheep.

Organ Culture

The mesenteric, mediastinal and parasternal lymph nodes and the spleen, thymus, liver, lung, myocardium, kidney and bone marrow were obtained from a foetus of a freshly slaughtered ewe. The tissues were washed in RPMI 1640 medium containing 1000 IU per ml of penicillin, cut into small pieces and attached to scarified plastic Petri dishes. Drops of medium were added to each piece to allow firm adherence and after a few minutes enough growth medium RPMI 1640 was added. The Petri dishes were left at 37°C in an incubator with an atmosphere of five percent carbon dioxide.

Primary Cell Cultures

Primary cell cultures were prepared from bovine foetal spleen, lung and kidney and from ovine foetal lung, spleen and bone marrow. The foetuses were withdrawn from freshly slaughtered pregnant cows or ewes. The organs were aseptically separated and placed in Hanks's balanced salt solution (HBSS) which contained pencillin and streptomycin. The parenchyma of the organs was cut into small pieces with scissors and then placed in a conical Erlenmayer flask with four volumes of 0.25 percent trypsin. This was left at $37^{\circ}C$ for one hour or at $4^{\circ}C$ overnight with continuous magnetic stirring. The supernatant fluid was collected and centrifuged at 250g to remove trypsin after adding more medium. The cells were then resuspended in medium 199 with 10 percent foetal calf serum and 100 IU of penicillin per ml and 2.5 ml of 4.4 percent sodium bicarbonate per 100 ml. The viability of the cells was determined using the Negrosin exclusion technique (Hanks and Wallace, 1958). The cells were diluted to give a final concentration of 5×10^5 viable cells per ml and seeded either in 75 cm^2 plastic bottles (Falkons Dickinson and Co. Ltd.) or in tubes with micro-coverslips. After the establishment of primary cell monolayers the cells were detached with a saline-trypsin-versene (STV) mixture. Cells thus established were mixed with ten percent DMSO and stored in the vapour phase of a nitrogen refrigerator at $-114^{\circ}C$.

Leucocyte Cultures

Attempts to propagate C. phagocytophila in peripheral leucocytes were carried out in the following ways: primary monocytic

cultures, macrophage cell lines derived from peripheral blood and whole blood cultures.

Monocytic cultures. The monocytic cultures were set up as described by Nyindo, Ristic, Huxsoll and Smith (1971) for canine monocytes with some modifications. Briefly, blood was collected from infected or uninfected sheep into heparinized containers. The buffy coat was separated by centrifugation and the erythrocytes lysed by adding 0.83 percent ammonium chloride solution. After three washings with HBSS the cells were resuspended in medium 199 or RPMI 1640 with HEPES buffer containing 20 percent autologous plasma to give a final concentration of 10^6 cells per ml and seeded in plastic bottles and tubes containing micro-coverslips. The medium was changed after 24 hours of incubation at 37°C with growth medium containing 20 percent FCS, LS or autologous serum. The medium was regularly changed at three to four day intervals and the cells monitored for growth and transformation into macrophages.

"Macrophage" cell lines. Peripheral blood of sheep and cattle was obtained and processed as described earlier. Alternatively the whole blood was layered over Ficol-Paque as described by Wardley, Lawman and Hamilton (1980) and the mononuclear cells separated. The cells were washed three times with HBSS and resuspended in minimum essential medium, medium 199 or RPMI 1640 containing 20 percent autologus plasma. The cells were diluted to approximately 10^6 cells per ml and distributed into flasks and tubes and incubated at 37°C. After 24 hours the medium was changed with growth medium containing either 20 percent FCS, LS or autologous

serum. Media were regularly changed at three to four day intervals and monitored regularly for growth and visible colony formation.

Whole Blood Cultures

Blood from sheep infected with C. phagocytophila were collected in sterile containers coated with heparin and cultured as follows:

Culture medium. Medium 199 with Earle's salts, 25 mM HEPES buffer and L-glutamine was commercially supplied. Ten percent foetal calf serum and 100 IU of penicillin per ml were added immediately before use.

Culture technique. In one series of experiments one ml of infected blood was mixed with three ml of medium in disposable polystyrene bottles. The bottles were left at 37°C either stationary or under continuous agitation for four, eight or 24 hours. Controls were left at 4°C for 24 hours. In a second series of experiments 0.1 ml of infected blood was mixed with 0.9 ml of blood collected from susceptible sheep with three ml of medium and incubated at 37°C for 24 hours. In a third series of experiments 0.5 ml of plasma from infected blood was added to one ml of blood collected from susceptible sheep and three ml of medium and incubated at 37°C for 24 hours.

Sub-cultures. To 0.9 ml of blood collected from susceptible sheep was added 0.1 ml of infected blood cultured for 24 hours and three ml of medium. Up to four blind passages were carried out.

Enumeration. Leucocytes were counted electronically (Coulter Electronics Ltd.). Infection rates were estimated from Giemsa

stained smears of blood before and after culture. When the number of infected cells was low, acridine orange stain was used. Cells containing one or more inclusions were recorded as infected and the infection rate expressed as percentage of total blood leucocytes. For the estimation of the number of organisms per infected cell the number of particles in 20 infected cells were estimated before and after culture.

Tick Organ Cultures

Snodgrass (1974) attempted to cultivate C. phagocytophila in organ cultures of Rhipicephalus appendiculatus without success. He speculated that the organism might adapt to organ cultures of its natural host Ixodes ricinus. The availability of limited numbers of I. ricinus prompted me to attempt to cultivate the organism in tick organ explants. The ticks were kindly provided by Dr. A. Walker and dissected by Miss L. Bell. The nymphs were maintained by feeding them on rabbits and they were dissected when the imago of the developing adult was starting to be visible. The organ cultures were maintained in Leibovitz L-15 medium supplemented with ten percent tryptose phosphate broth and ten percent FCS with or without 100 IU of penicillin per ml. They were kept in Leighton tubes with micro-coverslips and left at 24°C. They were regularly inspected for cell growth, media being changed every seven days.

Inoculation of Cultures with C. phagocytophila

One of the three infective inocula was used. They were allowed to adsorb after decanting existing media, for two hours but in some

instances they were left for 24 hours after adding minimum amounts of media. "Macrophages", spleen and lung cells were also co-cultured with infected leucocytes.

Tests for Infection

Coverslips were withdrawn at regular intervals and stained with Giemsa or acridine orange. Some coverslips were fixed in acetone for ten minutes and stained with anti-TBF conjugate as described in Chapter Five. From the organ cultures, squash smears were made and stained with anti-TBF conjugate after fixation with acetone. When infections were suspected blind passages were carried out and some suspected cultures were scraped and the suspensions inoculated into susceptible sheep.

Histological sections of organ cultures were made before and after culture and stained with Giemsa and anti-TBF conjugate. Some sections were prepared for electron microscopy.

Statistical Analysis

Data were analysed by conventional methods. Values obtained before and after culture were compared by analysing their pair-differences by Student's t-test.

RESULTS

Egg Inoculations

No evidence of propagation of the organism in the yolk sacs of embryonated hen eggs was obtained. Smears of yolk sac were negative when stained with anti-TBF conjugate and sheep inoculated

with yolk sac suspensions did not react clinically.

Organ Cultures

No organisms were demonstrated with Giemsa and acridine orange staining or with the fluorescent antibody technique. Some rickettsia-like structures were observed in squash smear preparations from lymph nodes 48 hours after culture but they were not infective to sheep. To investigate possible sites of multiplication other than the peripheral blood organ cultures were set up from all the tissues mentioned from a sheep which was sacrificed 48 hours after being inoculated with Cytoecetes phagocytophila. There was some evidence of the presence of C. phagocytophila in the fresh histological sections of the lymph nodes but no evidence of multiplication of the organism in any of the tissues was obtained after culturing them for 72 hours. Sheep inoculated with material from all of the tissues after 96 hours of culture failed to react clinically and were still susceptible to challenge inoculations. Electron micrographs did not reveal the presence of C. phagocytophila.

Primary Cell Cultures

None of the primary cell culture systems supported the growth of C. phagocytophila.

Leucocyte Cultures

Monocytic cultures. Monocytes derived from infected sheep or uninfected sheep were transformed into big vacuolated cells or multinucleated giant cells after one or two weeks of culture. When monocytic cultures from infected sheep were regularly observed

I found that some of them contained particles with morphological and staining characteristics resembling that of C. phagocytophila (Figure 4). Single particles and aggregates of particles initially appeared around the nucleus and eventually throughout the cytoplasm after seven to 13 days of culture. No such particles were observed in monocytic cultures obtained from uninfected sheep. When the infected cells were scraped and co-cultured with monocytic cultures from uninfected sheep similar particles were observed after four days. However, attempts to infect sheep with material obtained from such cells were not successful.

"Macrophage" cell lines. "Macrophage" cell lines were successfully established from the peripheral blood of 13 sheep and two cattle. The granulocytes and monocytes attached to glass surfaces after two to four hours. After four to seven days the granulocytes detached leaving the monocytes attached. Continued culture resulted in the multiplication of the mononuclear cells. The monocytes took a stellate or round appearance and during the second week some of them became multinucleated while others multiplied very fast forming colonies which became macroscopically visible (Figure 5). The formation of the colonies took place between two and eight weeks of culture, most of them occurring after two to four weeks. Trypsinization of the colonies and subculture resulted in the formation of confluent monolayers within seven days. Subsequent sub-cultures formed monolayers within three to four days.

Five of the ovine cell lines were derived from infected sheep but none of them harboured the organism. The "macrophage" cell

lines did not appear to support the propagation of C. phagocytophila. In some instances infected leucocytes were co-cultured with "macrophages" without causing infection. Infected leucocytes were observed lying in close attachment with macrophages without an apparent transfer of infection after 24 or 48 hours (Figure 5).

Whole Blood Cultures

When whole blood from infected sheep was incubated at 37°C with HEPES buffered medium 199, a significant increase in the numbers of infected cells occurred within four hours (Tables 6 and 7 and Appendix Table 1). Incubating infected blood for eight or 24 hours resulted in further increases. The percentage of infected cells rose from 27.00 ± 12.59 before culture to 33.22 ± 15.33 after four hours, 42.44 ± 17.41 after eight hours and 43.67 ± 14.15 after 24 hours of culture. Controls left at 4°C for 24 hours had a mean infection rate of 15.1 ± 10.24 percent. There was no significant difference between cultures which were left under continuous agitation and those left stationary ($t_8 = 0.14$, $p > 0.90$) (Appendix Table 2). There were significant differences between the percentage of infected cells of samples cultured for four hours and those cultured for eight hours ($t_9 = 3.47$, $p < 0.010$) but there were no statistically significant differences between those incubated for eight hours and those incubated for 24 hours ($t_9 = 0.68$, $p > 0.50$) (Table 7 and Appendix Table 1).

When infected blood was mixed with blood collected from susceptible sheep and incubated at 37°C for 24 hours, statistically significant increases in the percentages of infected cells were

recorded ($t_{13} = 5.16$, $p < 0.001$; Appendix Table 3) but when plasma was added to normal, uninfected blood no infected cells were observed after 24 or 48 hours.

Attempts to subculture incubated blood by mixing it with blood from susceptible blood gave inconclusive results. Three successive subcultures were achieved on four occasions but all died at the third passage. A susceptible sheep inoculated with a second passage reacted positively and typical organisms of C. phagocytophila were demonstrated in the neutrophils.

Attempts to assess the differences in the infectivity of fresh infected blood and infected blood cultured for 24 hours were made by a rough titration of infected blood before and after culture. For this purpose susceptible sheep were inoculated with dilutions of infected blood before culture and 24 hours after culture. Out of four samples tested no differences between the infectivity levels of fresh infected blood and cultured blood were observed in all but one sample in which the cultured blood's infectivity appeared to be higher by 100 ID_{50} (50 percent infective doses) (Table 8).

Increases in the number of organisms per infected cell as well as a marked change in the predominant morphological types were observed following culture. Twenty infected cells were studied from each sample before and after culture and the number of organisms per cell recorded. From 13 infected blood samples studied there were 45.23 ± 11.44 single particles per 20 infected cells and 3.15 ± 4.95 clusters per 20 infected cells before culture. After culturing infected blood for 24 hours there were 61.23 ± 30.60 single particles and 29.00 ± 12.11 clusters per 20 infected cells.

The increase in the number of clusters was very significant ($t_{12} = 6.48$, $p < 0.001$) the increase in single particles was also statistically significant ($t_{12} = 2.54$, $p < 0.050$) (Appendix Table 4).

Tick Organ Cultures

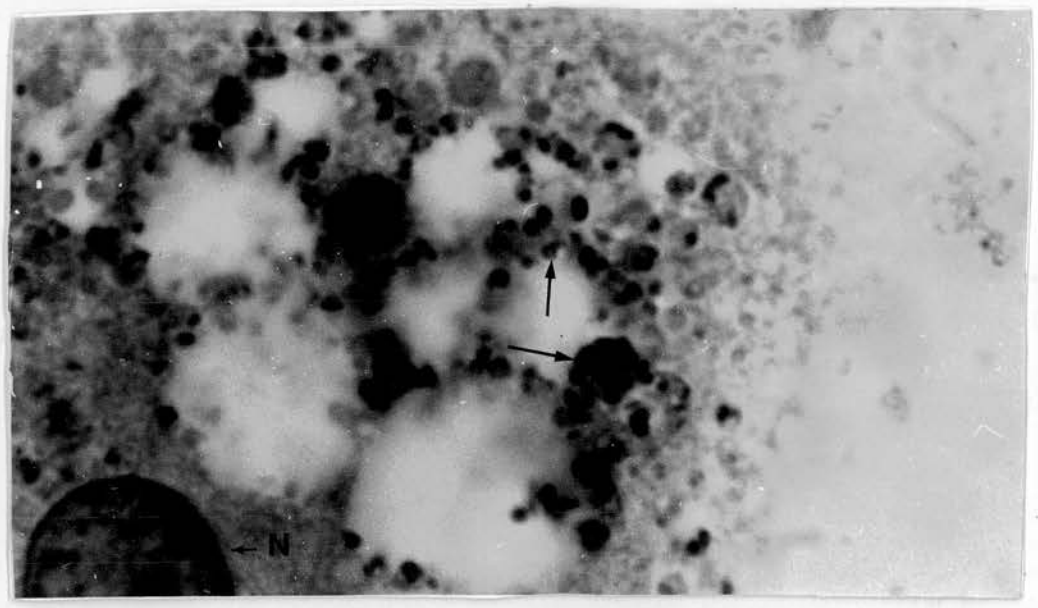
Only few organ cultures of the tick Ixodes ricinus were set up. Some of the organ cultures had proliferating cells but the numbers were too small to make enough replicates. No evidence of infection of tick cells was obtained in the few attempts of infecting them with C. phagocytophila.

Figure 4 Monocytic cultures infected with C. phagocytophila

A - Single and clusters of particles in the cytoplasm at the periphery of the nucleus (N) x 1800

B - Two big clusters near the nucleus (N) x 1800

C - Clusters and single particles throughout the cytoplasm x 1800



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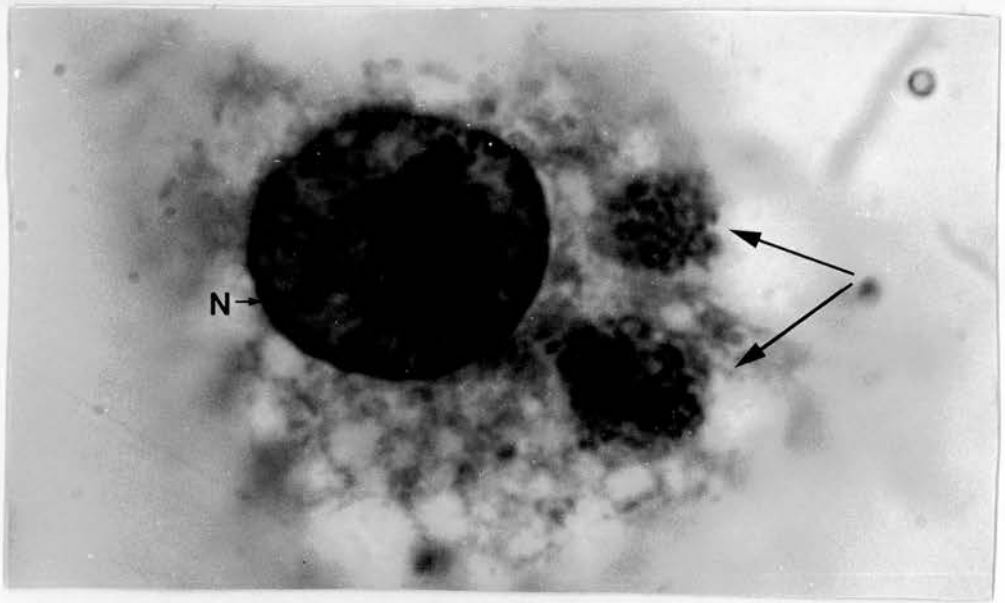

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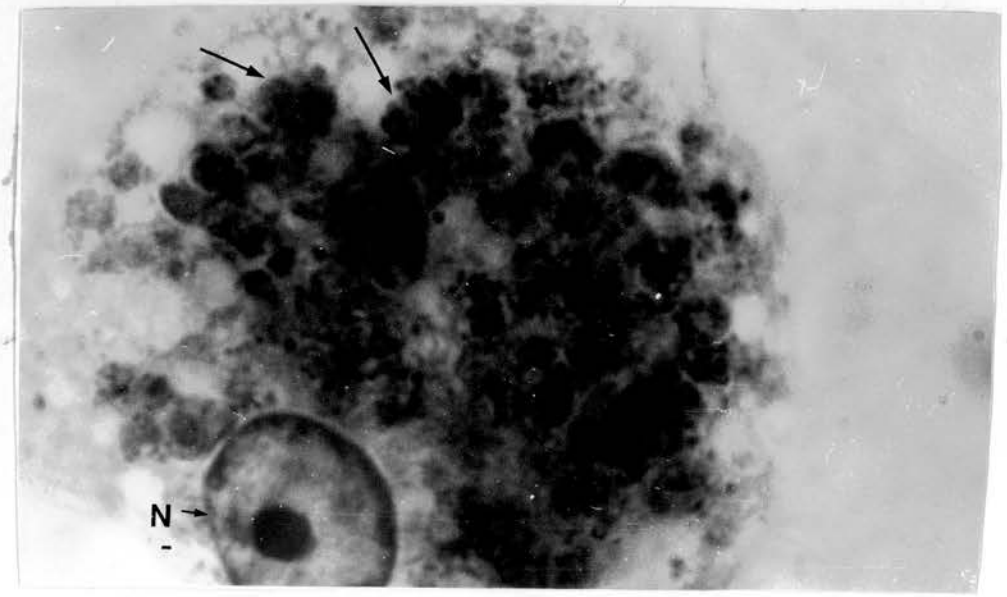


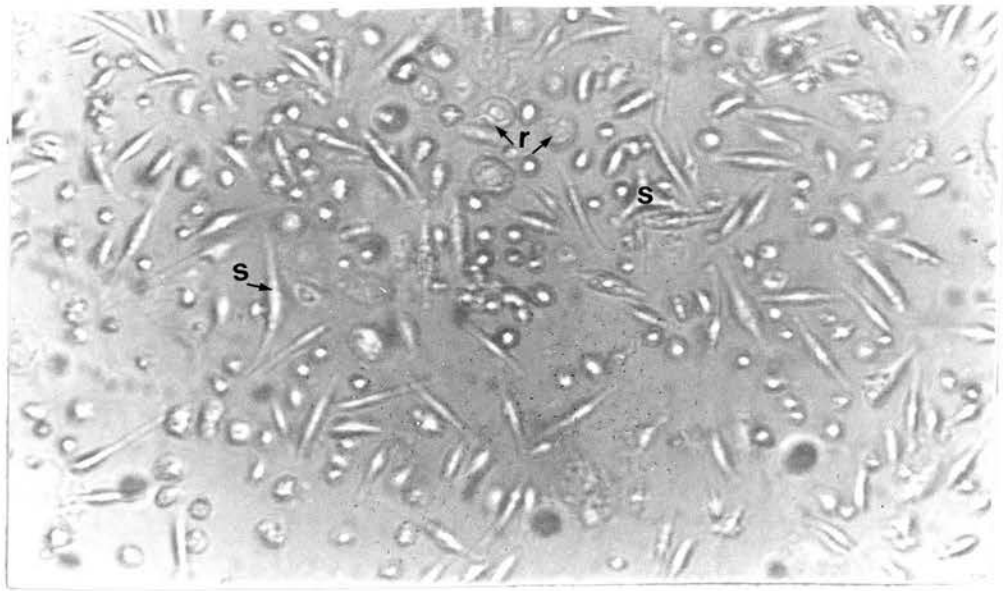
C

Figure 5 "Macrophage" cell lines

A - Fibroblast-like or stellate cells (s) and round (r) cells a week after culturing peripheral leucocytes of sheep x 200

B - Macroscopically visible colonies formed two weeks after culturing peripheral leucocytes of a cow

C - Co-culture of "macrophage" cell lines and infected leucocytes. An infected (I) polymorphonuclear leucocyte (PMN) is lying in the midst of three "macrophages". Nucleus (N) of three "macrophages" x 1800



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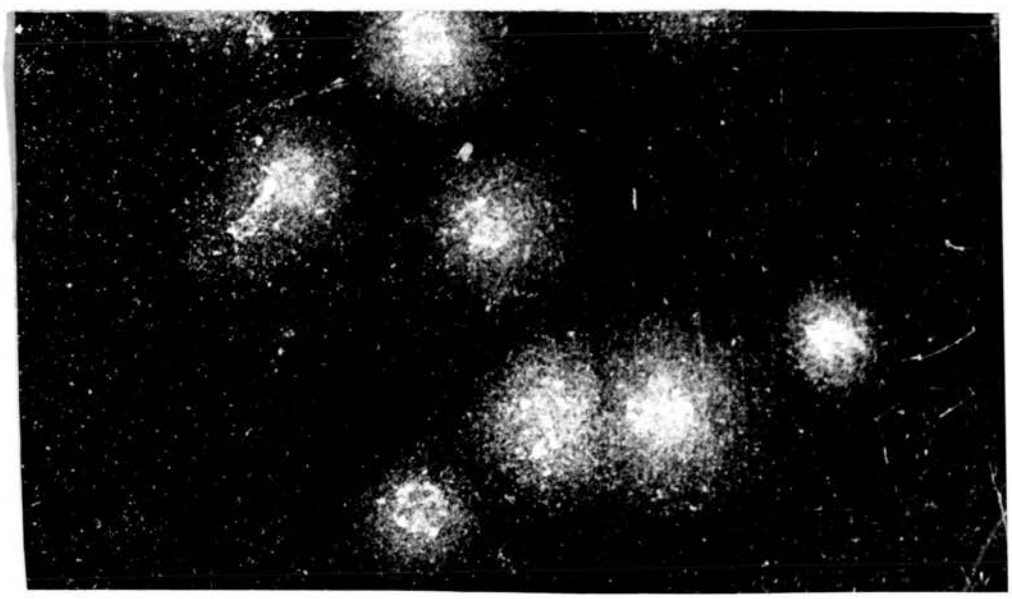

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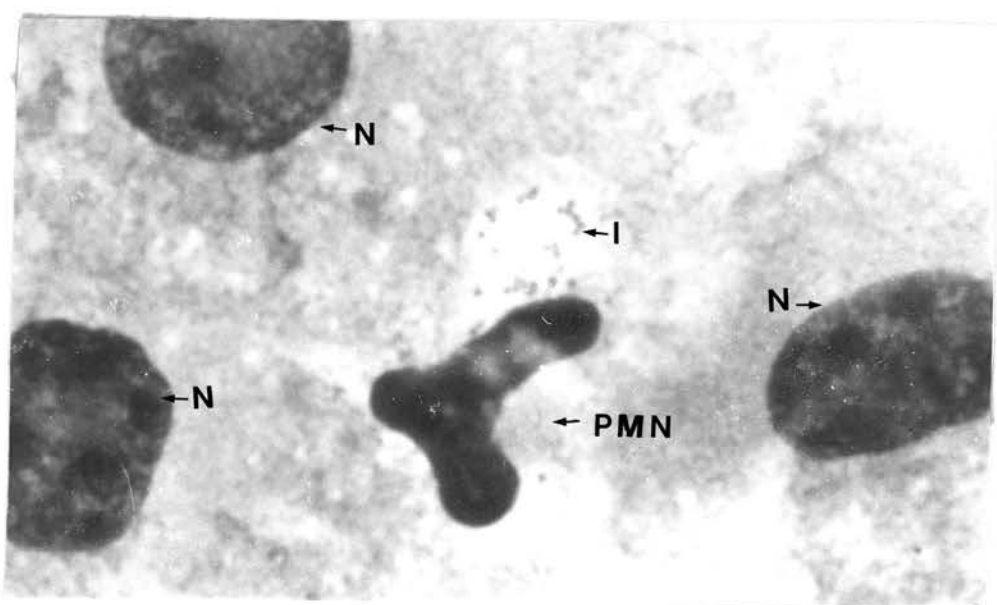


C

5

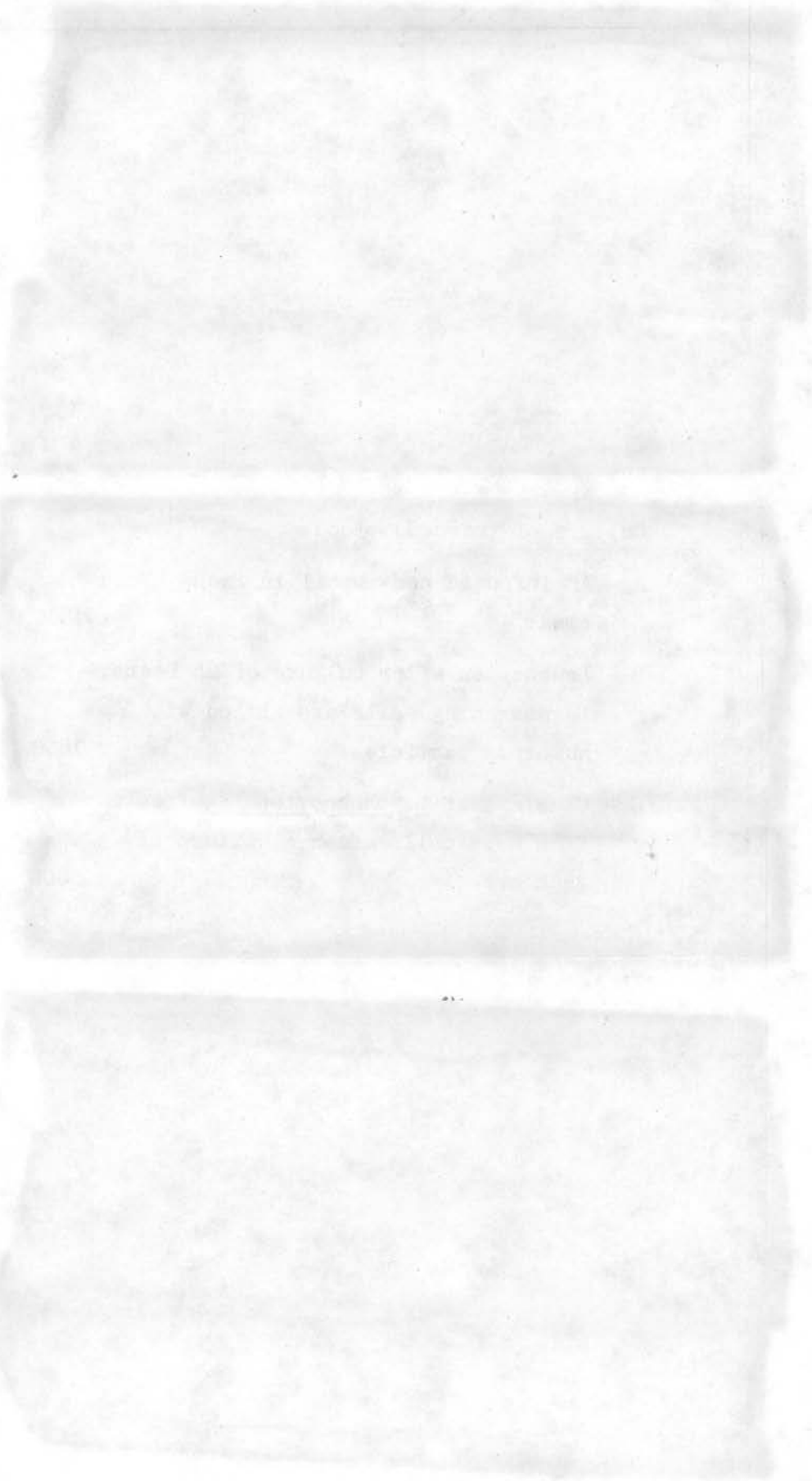
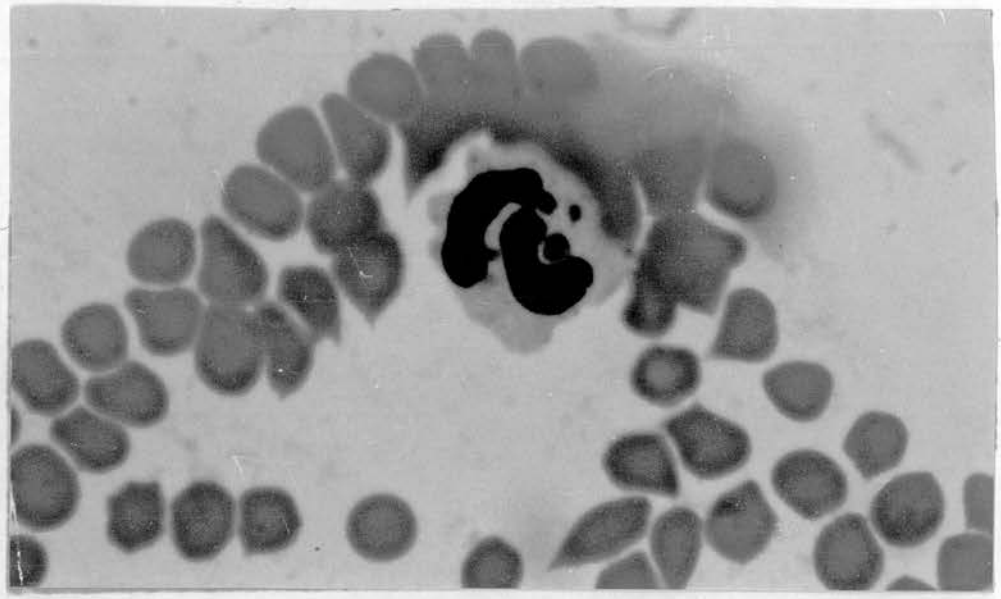


Figure 6 Culture of infected blood

A - An infected neutrophil in fresh blood smear x 1800

B - Leucocytes after culture of 24 hours. Degenerating cells are filled with numerous particles x 1800

C - Clusters of C. phagocytophila free from host cells after a culture of 24 hours x 1800



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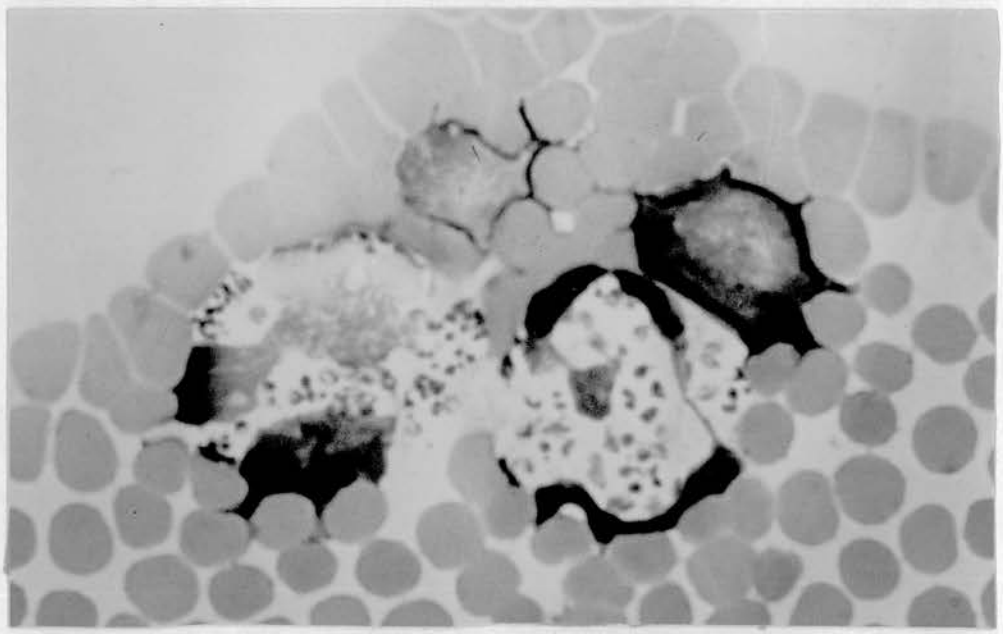

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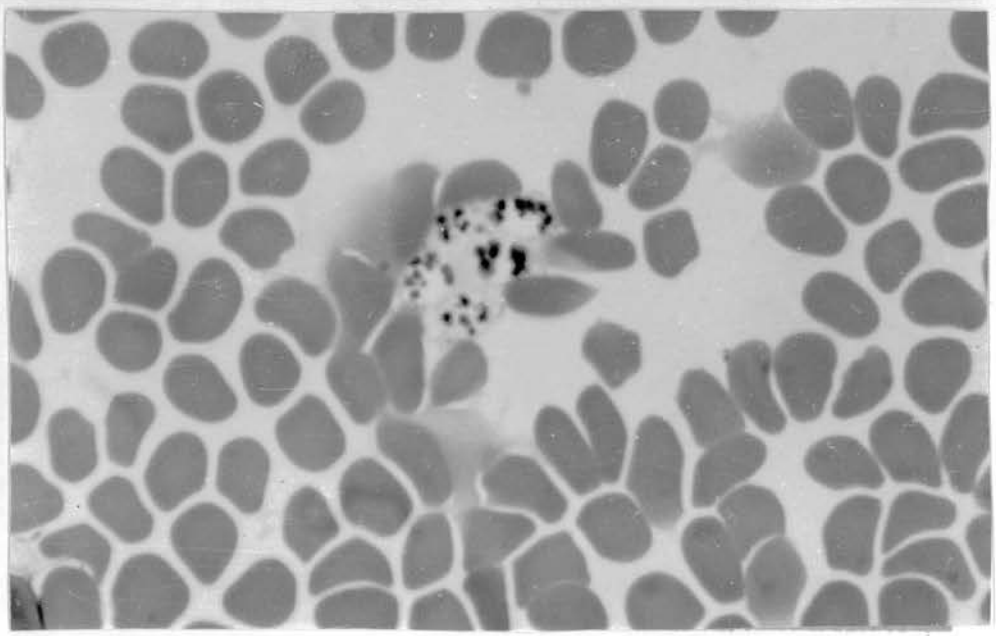


C

6

Table 6 Percentage of cells infected with C. phagocytophila before and after culture

Hours at 37°C	Number of observations	Mean	Standard deviation
0	9	27.00	12.59
4	9	33.22	15.53
8	9	42.44	17.41
24	9	43.67	14.15
24[a]	9	15.10	10.24

[a] at 4°C

Table 7 Significance of the differences in the number of infected cells before and after culture

Hours	$t_{(8)}$
0-4	3.75**
0-8	4.70**
0-24	5.93***
4-8	3.47**
8-24	0.68
4-24	3.67**

*** $P < 0.001$; ** $P < 0.010$

Table 8 Infectivity titres (ID_{50}) of infected blood before and after culture

Sample	Fresh	Cultured
120	10^5	10^7
158	10^6	10^6
165	10^4	10^4
166	10^6	10^6

DISCUSSION

Cytoecetes phagocytophila hitherto, has not been propagated in cell culture systems (Thrusfield et al., 1978). Two other animal rickettsias, Ehrlichia canis and Neorickettsia helminthoeca, however, have been propagated in monocytic cultures (Nyindo et al., 1971; Brown, Huxsoll, Ristic and Hilderbrandt, 1972). Although these organisms are similar to C. phagocytophila their target cells are the mononuclear cells of the dog whereas the main target cells of C. phagocytophila are the neutrophils. Ehrlichia equi whose target cells are the neutrophils has not been propagated in vitro.

In the present study the organism was not propagated in primary cell culture systems or organ cultures but particles similar to C. phagocytophila were consistently observed in the cytoplasm of macrophages obtained from mononuclear cells of infected sheep. However they were not infective to susceptible sheep. Scott and Munro (1975) had observed similar particles on leucocyte cultures derived from sheep infected by C. phagocytophila and found them non-infective to sheep. The failure of such organisms to infect sheep raises some questions as to their nature. Does the organism lose its infectivity after being adapted to in vitro growth or are there other rickettsia-like organisms in the leucocytes of infected sheep? A weakness of the present study was that monocyte cultures were not set up from leucocytes of infected sheep at later stages of infection when the monocytes

are expected to be infected (Chapter Two).

Thrusfield and his colleagues (1978) attributed past failures to propagate C. phagocytophila to the fact that the cells predominantly parasitised by the organism were the granular leucocytes which have not yet been cultured in vitro. Indeed, I found in this study that the leucocytes started to degenerate very rapidly, the total counts as estimated by an electronic counter being significantly reduced within 24 hours ($t_8 = 5.00$, $p < 0.01$; Appendix Table 2). Despite this, however, the organism appeared to continue to multiply in apparently degenerating cells (Figure 6). On many occasions groups of organisms were found with little or no host cell material (Figure 6).

The increase in the percentage of infected cells when infected blood was mixed with blood from susceptible sheep suggests that new cells had been invaded. On the other hand, when plasma was mixed with normal blood no infected cells were observed. The latter finding suggests that the cell-free form is too fragile to initiate infection in vitro and the increases in the number of infected cells were not due to new cells being invaded but due to the continued multiplication of the organisms in already infected cells, thus rendering infected cells easily detectable.

In the process of attempting to propagate the organism in monocytic cultures, I discovered a new system of establishing cells lines from the peripheral blood of sheep and cattle. These "macrophage" cells, however, did not support the growth of the organism.

Despite the fact that the cell lines derived from peripheral monocytes did not apparently support the *in vitro* propagation of *C. phagocytophila*, the establishment of the cell lines by itself was important. Nunn and Johnson (1979) stressed that establishing cell lines from the peripheral blood had immense advantages for the propagation of other organisms and viruses but it was very difficult. When circulating monocytes were cultured for prolonged periods some of them transformed into fibroblast-like or multinucleated, giant cells. These cells supported the growth of viruses (Moore, Redmond, Katada and Wallace, 1970) and rickettsia (Nyindo *et al.*, 1971; Brown *et al.*, 1972). Greenwood (1973) reported that sheep blood monocytes cultured in frequent changes of 100 percent autologous plasma grew and divided by mitosis to form large macrophages but mitotic activity died out having gradually declined after eight days of culture. Recently, Nunn and Johnson (1979) reported that they had established cell lines from porcine blood and Wardley and his colleagues (1980) reported the successful establishment of cell lines from no less than seven species. The method described in the present study was simpler than that described by Nunn and Johnson (1979) and Wardley and his colleagues (1980). It did not require the separation of mononuclear cells by density gradient centrifugation. I also found that while ovine cells readily grew with FCS or LS, the bovine cells formed dividing colonies only if 20 percent autologous unheated serum was used. Furthermore, I found that although ovine and bovine granulocytes and monocytes adhered to glass or

plastic surfaces with or without the addition of plasma, the use of autologous plasma for the first 24 hours of culture enhanced their attachment and increased the success rate of establishing colonies. Most colonies were formed after two weeks of culture but some colonies were formed as early as one week after setting up the cultures or as late as eight weeks. It is difficult to understand what triggers the formation of rapidly growing colonies after the primary monocytic cultures were established and in some cases after extensive giant cell formation had taken place. The colonies were either scraped with a rubber policeman or detached with STV. Most cells readily detached with STV but some were difficult, needing higher concentrations of trypsin.

I did not attempt to investigate the origin of these cells but it is unlikely that they originated from cells other than the monocytes. The observation of fibroblast-like cells in macrophage cultures of monocytic origin have led some workers to speculate that the monocytes might differentiate to macrophages and to fibroblasts while others have contended that the fibroblast-like cells in macrophage cultures were due to contamination with fibroblasts (Volkman, 1970). Rangen (1967), for example, argued that the spindle cell or fibroblast-like colonies observed in chicken macrophage cultures obtained from the buffy coat might be attributable to contamination by connective tissue during collection of blood samples. Wardley and his colleagues, on the other hand, did not explain whether the "macrophage" cell lines they established from seven species had fibroblast-like appearancee

as I frequently observed. They reported that the cells possessed chemical, structural and immunological characteristics of macrophages. In the present study, I did not undertake to characterise these cells chemically or immunologically, but from the morphological point of view they appeared to have retained their phagocytic characteristics.

The lack of *in vitro* methods for the propagation of *C. phagocytophila* has hampered further studies on the biological characterisation of the organism. I believe that whole blood cultures will be useful in the morphological characterisation of the organism and in improvement of antigen yields for immunological studies (Chapters Four and Six).

CHAPTER FOUR

DEVELOPMENT OF *CYTOECETES PHAGOCYTOPHILA*

INTRODUCTION

The pleomorphism of C. phagocytophila is well recognised and fully documented. Early workers at the Moredun Institute, Edinburgh, drew attention to the similarities between the forms of C. phagocytophila and the causative agent of psittacosis and postulated a developmental cycle similar to that advocated by Bedson and Bland (1932) for the agent of psittacosis (Foggie, 1951; Gordon et al., 1962). However, they and other workers (Tuomi and von Bonsdorff, 1966) failed to establish a developmental cycle.

In experiments designed to study the in vitro propagation of C. phagocytophila, I noticed that the predominant forms of the organism after culture of infected blood for 24 hours markedly differed from those of fresh blood samples. Experiments were, therefore, designed to pursue this observation further.

MATERIALS AND METHODS

C. phagocytophila

The Old Sourhope (OS) strain was handled and used as described in Chapter Two.

Sheep

Eight susceptible adult sheep were inoculated with C. phagocytophila and blood samples in heparin taken every day for 21 days. The samples were divided in two and one of them cultured overnight as described in Chapter Three.

Light Microscopy

Blood smears were prepared from each sample before and after

culture and stained with Giemsa. Ten infected cells from each sample were studied and the type of inclusions in each infected cell classified. The infected cells were then divided into three groups. Those which contained only discrete particles were classified in Group One. Those with clusters of particles were included in Group Two and those which contained discrete particles and clusters were included in Group Three. No attempts were made to classify discrete particles into the so called 'initial bodies' and 'elementary bodies' and the clusters into 'morulae','fragmenting forms', 'ring forms', etc. (Gordon et al., 1962). Instead, inclusions with apparently no more than one particle, irrespective of size and shape, were regarded as discrete and the clusters represented inclusions with more than one particle.

Electron Microscopy

Buffy coat cells as free as possible from erythrocytes were prepared from fresh and incubated infected blood. They were fixed in 2.5 percent gluteraldehyde solution in 0.1M cacodylate buffer overnight, rinsed in 0.1M cacodylate buffer for five minutes three times and post-fixed in one percent osmium tetra oxide for 60 minutes. They were then rinsed again with 0.1M cacodylate buffer and dehydrated in progressively higher concentrations of ethyl alcohol. The cell pellet was treated with propelene oxide three times for ten minutes and left in a 1:1 propelene/Araldite (Ciba Ltd.) mixture for two hours and then left in 100 percent Araldite overnight and embedded in gelatine capsules in fresh Araldite and allowed to polymerize at 60°C for 48 hours. Sections were made

with Huxley ultra-microtome, stained with lead citrate and electron micrographs taken with a Philips EM 400 Electron Microscope.

Analysis of Data

Means and standard deviations were calculated according to conventional methods and pair-differences analysed by Student's t-test for significance.

RESULTS

Light Microscopy

The organisms in the cytoplasms of granulocytes and monocytes were as either discrete particles or clusters of particles. Many cells contained more than one inclusion. Fresh blood samples taken on the first day of parasitaemia had 77.5 ± 15 percent of the infected cells containing discrete particles, 7.5 ± 12 percent of the infected cells contained clusters of particles and 15 ± 11 percent of infected cells contained a combination of both. The pattern was the same throughout the period of parasitaemia except on the last day of parasitaemia when there was an apparent rise in the number of infected cells containing clusters of particles (Figure 7 and Table 9). In contrast, when infected blood taken on the first day of parasitaemia, was cultured for 24 hours only 10 ± 13 percent of the infected cells contained discrete particles compared to 55 ± 25 percent of infected cells containing exclusively clusters of particles and 35 ± 28 percent containing a combination of both. Cluster predominance in cultured samples continued throughout the period of parasitaemia (Figure 7 and Table 10).

The number of cells containing single discrete particles only, on the first day of parasitaemia, was significantly reduced after culture ($t_7 = 12.1$, $p < 0.001$) and the number of cells containing clusters was significantly increased ($t_7 = 5.00$, $p < 0.001$). The differences were significant throughout the period of parasitaemia (Appendix Tables 5 to 10). In six of the eight sheep studied, the organism was detected by culturing blood one day before it was detected from fresh blood smears and in seven of the sheep it was detected by culture for at least one more day after fresh blood smears were apparently negative (Appendix Tables 5 to 10).

Electron Microscopy

Cytoecetes phagocytophila was found in cytoplasmic vacuoles of granulotyes and monocytes. The vacuoles were surrounded by a single membrane and their sizes and shapes were variable. There were one or more particles of various sizes and shapes either closely associated to each other or widely dispersed in big vacuoles (Figure 8) but the vacuole around some particles was not clearly visible (Figure 9).

Each particle was surrounded by two membranes; the outer one had a rippled appearance. The particles were made of electron dense and lucent areas; the small particles had a dense core with a clearly identifiable nucleoid while the electron dense areas of the big particles was dispersed. On some occasions dumb-bell shaped particles, indicating binary fission were observed. Small particles were found outside an apparently intact vacuole (Figure 8). Most particles were intact but a few degenerated particles were

observed (Figure 9). It was not uncommon to find apparently empty vacuoles or vacuoles with very few small particles and "ghost" structures (Figures 8, 9 and 10).

Comparison between cells before and after culture was difficult because the cells tended to degenerate after culture (Chapter Three) but it was apparent that cultured cells had more vacuoles than fresh cells. In many instances, inclusions were found in apparently degenerated cells without losing their structural characteristics. Quantitative differentiation was not attempted because the number of particles observed depended on the manner by which the vacuoles were sectioned.

Figure 7 Comparison of the morphological types of *C. phagocytophila* in fresh and cultured blood samples

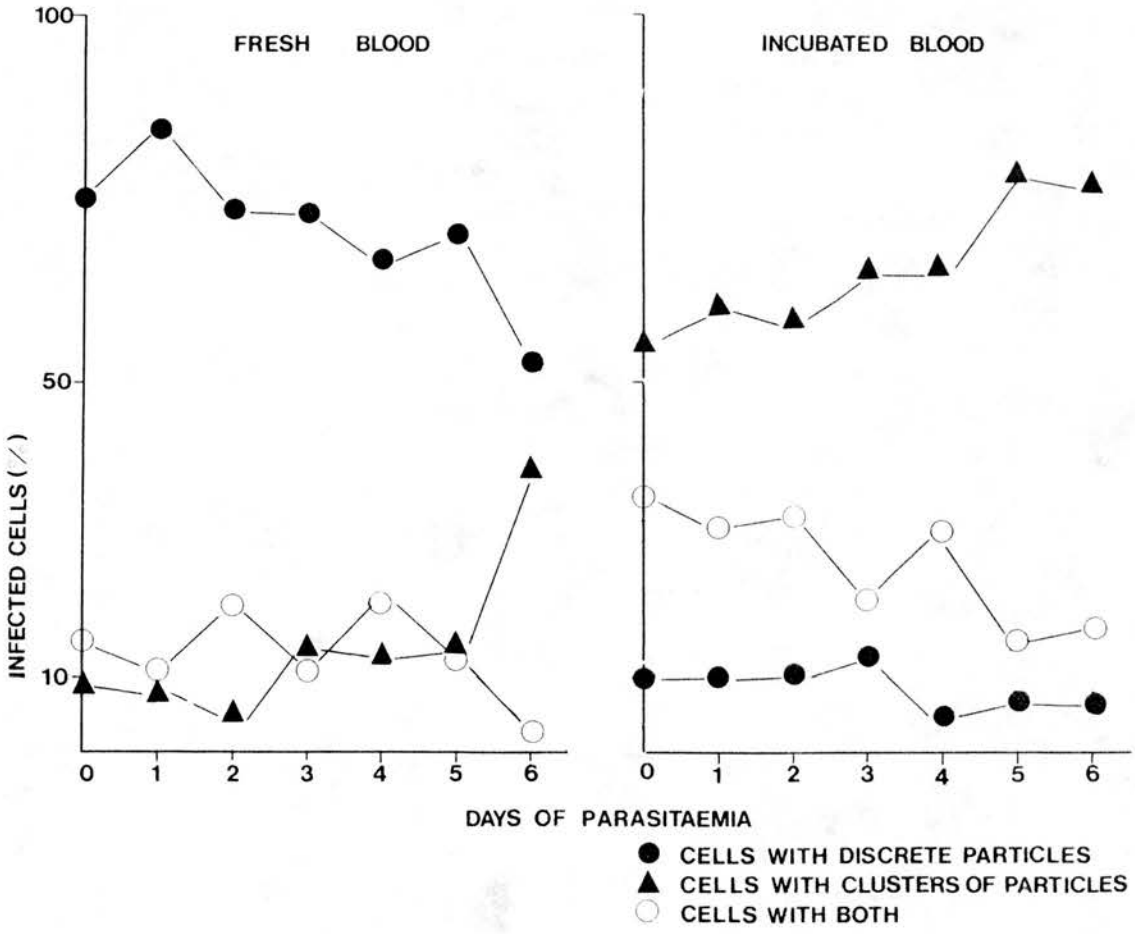

100
50
10
FRESH BLOOD
INCUBATED BLOOD
INFECTED CELLS (%)
0 1 2 3 4 5 6
0 1 2 3 4 5 6
DAYS OF PARASITAEMIA
CELLS WITH DISCRETE PARTICLES
CELLS WITH CLUSTERS OF PARTICLES
CELLS WITH BOTH

Figure 8 Proposed stages of development of

C. phagocytophila

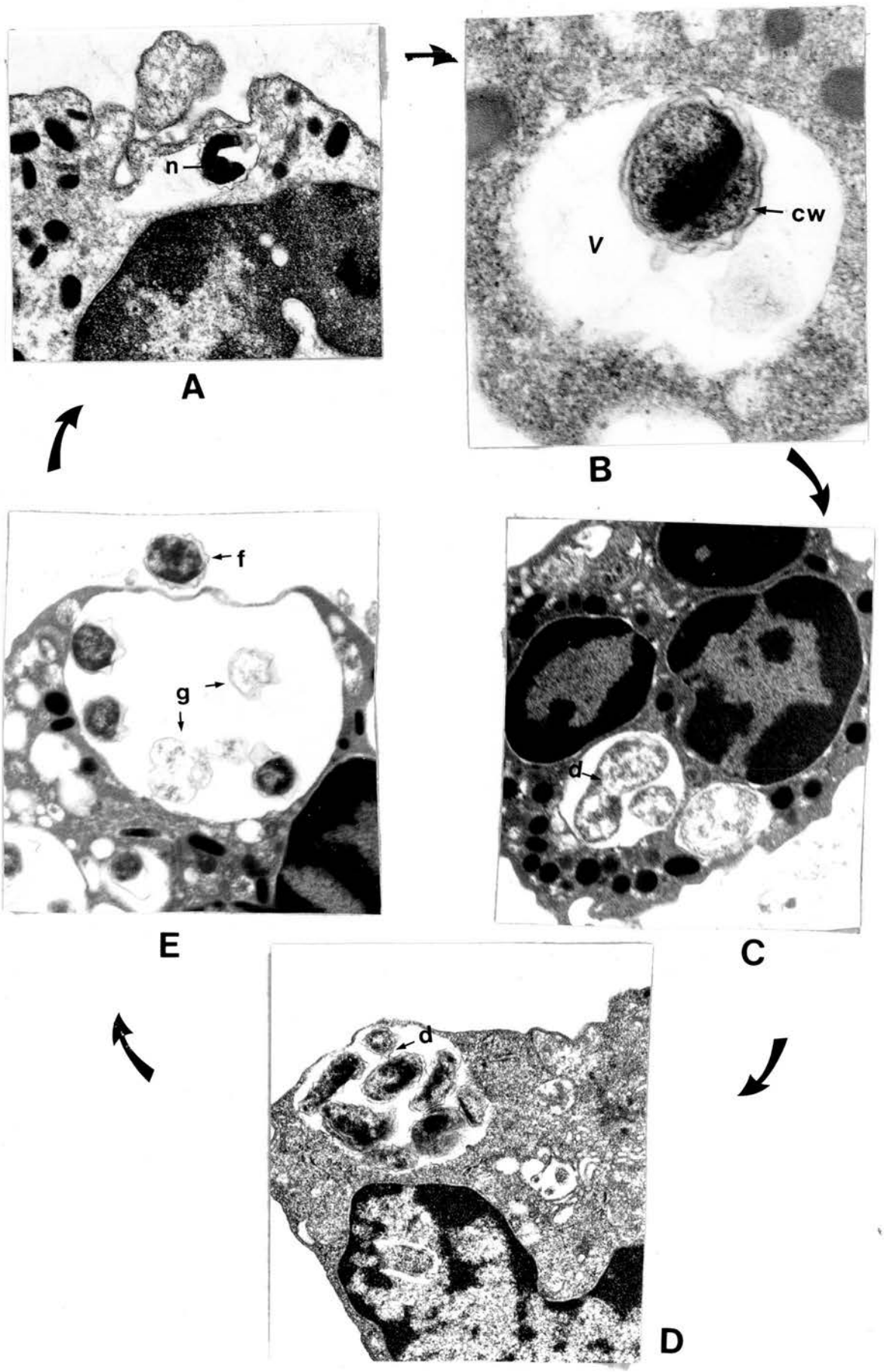
A - A small single particle with a distinct nucleoid (n) x 10000

B - A single particle in an enlarged vacuole (V). The nucleoid is still distinct but not detached. The particle is surrounded by a distinct cell wall (cw) made of two rippled membranes x 30000

C - A cell with two vacuoles. One vacuole contains two particles one of which is dumb-bell shaped (d) indicating division by binary fission x 10000

D - A cell with one cytoplasmic vacuole containing clusters of particles. An organism in the process of complete division is seen (d) x 10000

E - A big vacuole containing three small particles and two "ghost" structures (g). One small particle is lying free outside the vacuole (f) x 10000


n
A
cw
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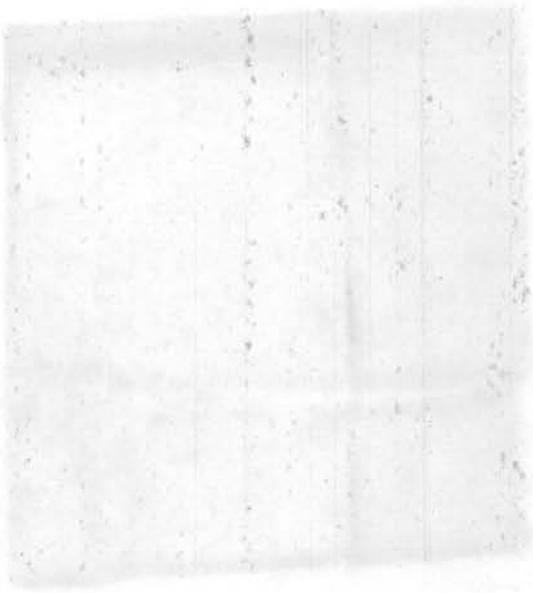
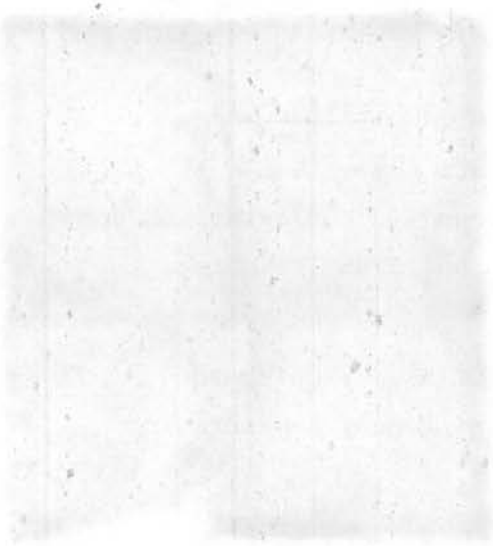
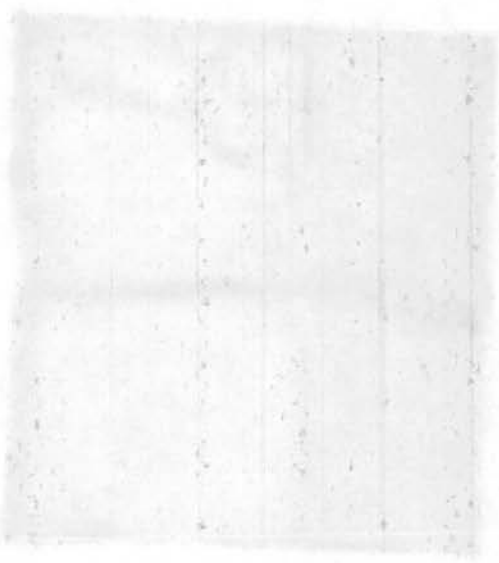
D

8







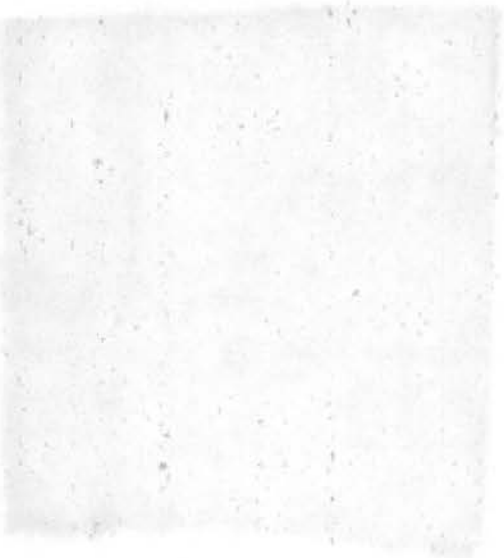
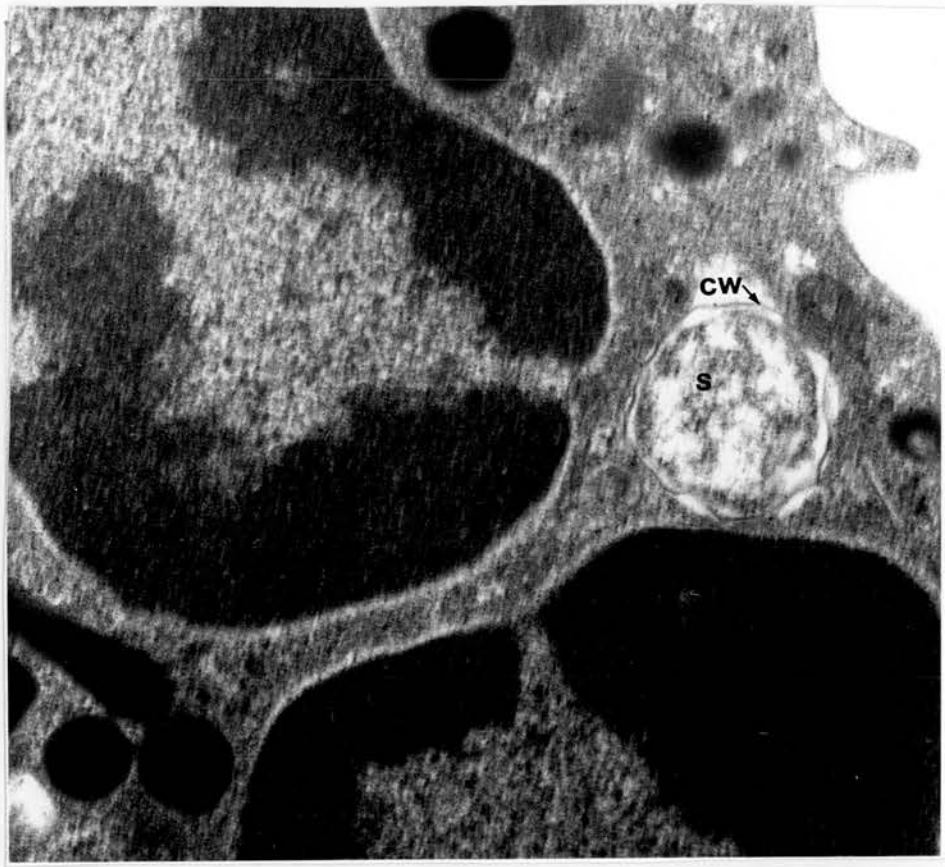


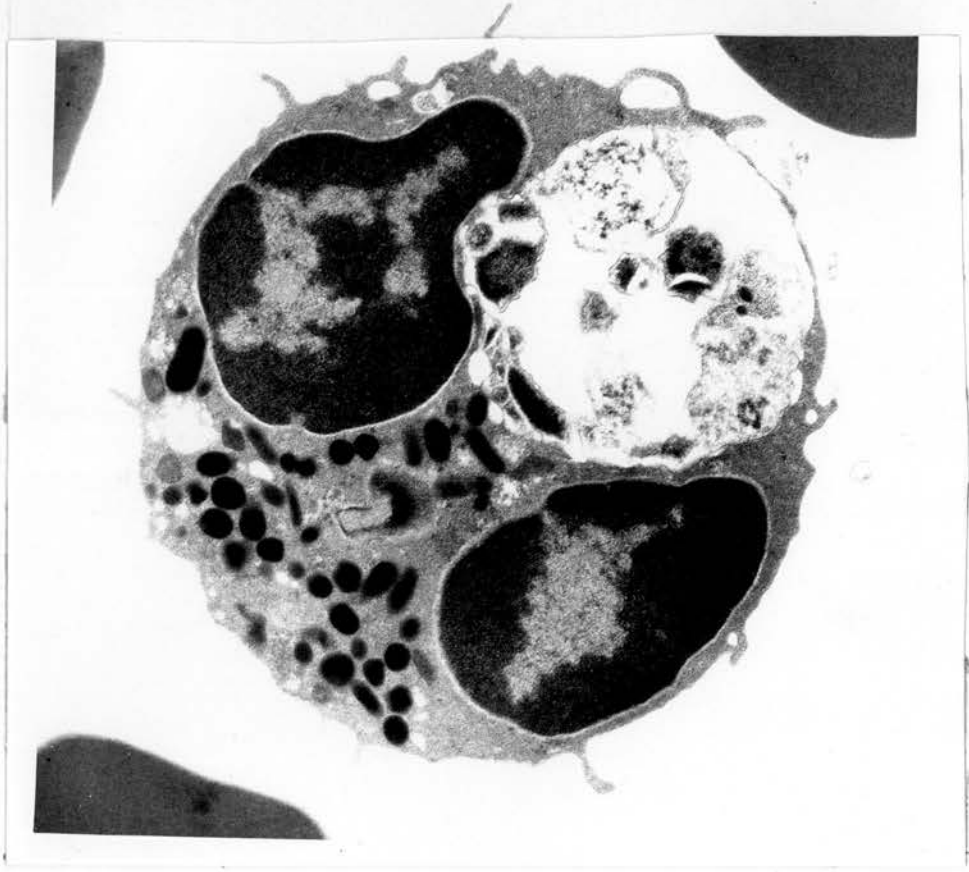
Figure 9 Ultrastructure of *C. phagocytophila*

A - A single particle (s) with a cell wall (cw) tightly bound to the cytoplasm and the vacuole is not clearly defined x 30,000

B - A granulocyte with a cytoplasmic vacuole containing many degenerating particles x 10,000



A



B

9

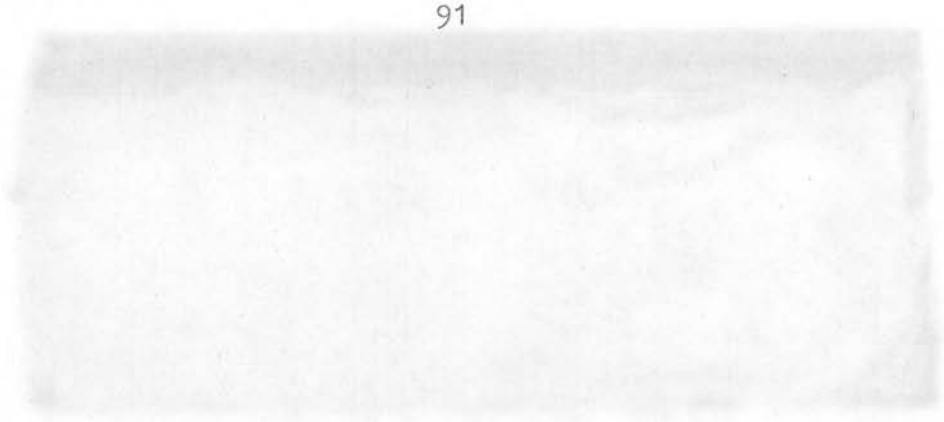


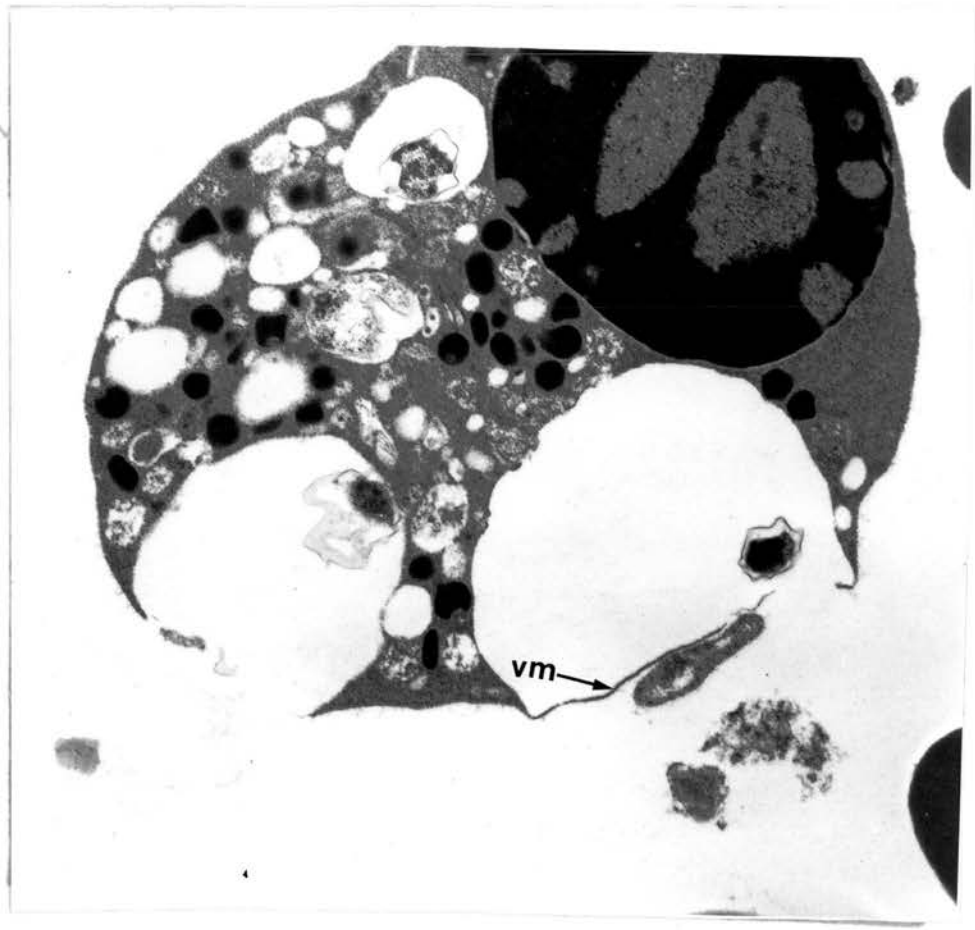
Figure 10 Ultrastructure of C. phagocytophila

A - A granulocyte with several cytoplasmic inclusions. The membranes of the vacuoles (vm) of two of the inclusions are broken

x 10,000

B - A granulocyte with four cytoplasmic inclusions. Some big particles contain small dense bodies (b) beneath them. One of the inclusions contains big "ghost" structures (g) and small dense particles. An elongated large particle without cell wall (f) is lying outside the cell

x 10,000



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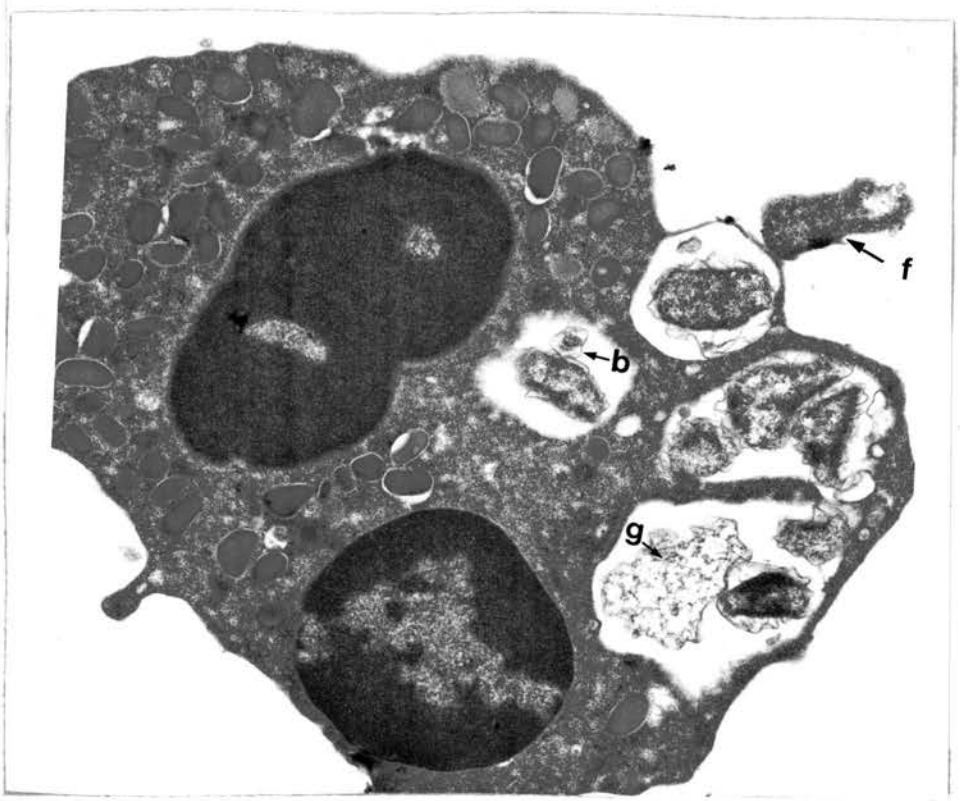


B

10

Table 9 Mean percentage of infected cells in fresh blood samples containing discrete particles, clusters or both

Day of parasitaemia	Number of observations	Discrete particles	Clusters	Both
-1	8			
0	8	77.5 ± 14.9[a]	7.5 ± 11.6	15.0 ± 10.7
1	8	80.0 ± 17.7	7.5 ± 10.4	12.5 ± 10.4
2	8	73.8 ± 22.6	3.8 ± 5.2	22.5 ± 22.5
3	8	72.5 ± 17.5	16.3 ± 7.4	11.3 ± 13.6
4	8	67.5 ± 21.9	13.8 ± 13.0	18.8 ± 14.6
5	8	71.3 ± 18.1	15.0 ± 13.1	13.8 ± 10.6
6	4	52.5 ± 41.1	47.5 ± 41.1	0
7	4	0	0	0

[a]Mean and standard deviation

Table 10 Mean percentage of infected cells in cultured blood samples containing discrete particles, clusters or both

Day of parasitaemia	Number of observations	Discrete particles	Clusters	Both
-1	6	43.3 ± 29.4[a]	45.0 ± 26.6	11.7 ± 14.7
0	8	10.0 ± 13.1	55.0 ± 24.5	35.0 ± 11.6
1	8	10.0 ± 10.7	60.0 ± 16.0	30.0 ± 16.9
2	8	10.0 ± 13.1	58.8 ± 24.2	31.3 ± 23.0
3	8	13.8 ± 14.1	66.3 ± 13.0	20.0 ± 10.7
4	8	5.0 ± 7.5	65.0 ± 22.7	30.0 ± 23.3
5	7	7.1 ± 7.6	78.6 ± 10.7	14.3 ± 5.3
6	8	6.3 ± 10.6	77.5 ± 14.9	16.3 ± 14.1
7	7	10.0 ± 14.1	80.0 ± 15.28	10.0 ± 14.1

[a]Mean and standard deviation

DISCUSSION

Attempts to characterise the organism morphologically have been made ever since the organism was demonstrated in the cytoplasms of granulocytes and monocytes (Foggie, 1951). The organism appeared to have at least two forms of single particles and two forms of groups of particles after staining with Romanowsky stains. Borrowing from the nomenclature used for the organisms now known as Chlamydia, early workers started calling the small single particles "elementary bodies", and the big single particles "initial bodies". The groups of particles made up of tightly packed small particles were named as "morulae" and those made up of loosely packed big particles were called "fragmenting forms", "clusters", "ring forms" etc. Demonstrating the assumed life cycle, however, has proven impossible. Foggie (1951), inoculated two sheep with plasma of sheep suffering from TBF and from the 48th hour onwards, he examined blood smears every three hours. In one sheep "initial bodies" were first seen at the 133rd hour "fragmenting forms" at the 139th hour and "morulae" at the 145th hour. He observed similar patterns in the other sheep, but no distinct change from one form to the next was observed, initial bodies predominating throughout the period of parasitaemia. In a delayed publication of experimental investigations on the possible cycles of development, Gordon and his colleagues (1962) reported that single, undivided forms predominated throughout the period of parasitaemia.

Since the sojourn of the mature granulocytes in the circulation is very brief, being only a few hours (Hirsch, 1972) it would be

difficult to follow the morphological changes that the organism might undergo in infected cells using the in vivo method favoured by Foggie (1951) and Gordon and his colleagues (1962). As infected cells leave the circulation new, uninfected, cells may arrive from the bone marrow reserve (Snodgrass, 1974; Chapter Six). An in vitro study eliminated the above obstacle and by limiting the study to a specific sample that is studied before and after culture, a better assessment of the changes is likely.

The findings in this study agree with the observations of Foggie (1951) and Gordon and his colleagues (1962) that the discrete bodies appeared first and predominated throughout the period of parasitaemia. However, when blood samples were incubated for 24 hours the situation was reversed; the clusters became dominant. These findings suggest that C. phagocytophila develops from single discrete particles to clusters of particles. The electron microscopic findings support this hypothesis.

The ultrastructural findings in this study generally agree with those described by Tuomi and von Bonsdorff (1966), but we differ in interpretation. Most of the particles were found in clearly defined vacuoles but the vacuole surrounding some single particles were not clearly discernible. Sells, Hilderbrandt, Lewis, Nyindo and Ristic (1976) reported similar observations on Ehrlichia equi. The particles tightly enclosed by a vacuole not clearly separated from the cytoplasm may represent early phases of infection when the organism is invaginated by the phagocyte. The organisms appeared to enlarge and divide by binary fission

(Figure 8). The large particles were less densely stained than the small particles (Figures 8, 9 and 10) and this might be due to the process of division when nucleoid material is dispersed.

Tuomi and von Bonsdorff (1966) postulated that the likely mechanism of entry to the cell was by phagocytosis and that the large particles were the most likely ones to escape the host cell. This hypothesis was based on their frequent observation of single large particles and lack of single small particles and their finding of what they claimed was extrusion of vacuoles with big particles. In this study not only were single, small particles frequently observed (Figure 8) but small particles were found outside vacuoles (Figure 8) and inside vacuoles which had apparently ruptured (Figure 10). From these observations it seems that the organisms leave the vacuoles gradually and neither by the bursting of tightly packed particles nor by the process of extrusion of the whole contents of the vacuoles as described by Tuomi and von Bonsdorff (1966). Few organisms appeared to have degenerated (Figure 9) but no evidence was obtained of the presence of lysosomal granules inside the vacuoles. No firm evidence of ways of multiplication other than binary fission were obtained but small particles lying beneath big ones and ghost structures similar to those described by Kraus, Davies, Odegaard and Cooper (1972) in Cytoecetes ondiri were occasionally observed (Figure 10).

It appears that the development of C. phagocytophila is simple and not as complicated as previously thought; the multiplication being by simple binary fission inside cytoplasmic vacuoles. By linking the light microscopic findings of the present

work and that of other workers (Foggie, 1951; Gordon et al., 1962) with the ultrastructure of the organism, I venture to propose the following (Figure 8):

Small particles of C. phagocytophila are phagocytosed by eosinophils, neutrophils and monocytes, in that order. The invaginating cell membrane tightly surrounds the organism but eventually enlarges to form a vacuole. The organism enlarges and divides by binary fission and depending on the size of the particles and how tightly packed the organisms are they appear under the light microscope as discrete particles or as big irregular masses. The vacuole increases in size and the number of particles in the vacuole also increase to give rise to what appear as "morulae" or clusters under the light microscope depending on the size of the particles. Some particles gradually leave the vacuole and are phagocytosed by other cells.

The taxonomic position of C. phagocytophila has been based on its mode of transmission and its morphological similarities to other rickettsias. The small particles have some similarities to the small particles of the Chlamydia but there is no evidence of a life cycle in C. phagocytophila. The organism also differs from the organism of the genus Rickettsia, except R. sennatsu, because they lack cytoplasic vacuoles (Anderson, Hopps, Barile and Bernheim, 1965). The present findings suggest that the development of the organism does not differ from that of other bacteria and rickettsia apart from its location in cytoplasmic vacuoles.

CHAPTER FIVE

SEROLOGICAL TESTS

INTRODUCTION

Specific antibodies to C. phagocytophila have been demonstrated by a direct fluorescent antibody technique (Tuomi, 1967[d]) and by a complement fixation test (Snodgrass and Ramachandran, 1971). However, the reproducibility of these tests has not been confirmed by other workers. Snodgrass (1974) and Koske (1976) found that the immunofluorescent test described by Tuomi (1967[d]) was difficult to reproduce because of intense non-specific fluorescence and Snodgrass (1974) emphasized that the CF test was not routinely used because the method of antigen production was not ideal.

The present study was aimed at making a comparison between these and other serological tests to choose the best for studying the humoral immune response of animals infected with C. phagocytophila.

MATERIALS AND METHODS

C. phagocytophila

The Old Sourhope strain was handled and used as described in Chapter Two.

Complement Fixation Test (CFT)

Reagents. Veronal buffer (VB) was prepared from commercially supplied tablets (Oxoid Ltd.). Complement was prepared from healthy adult guinea pigs and treated with Richardson's reagent (Richardson, 1941; Appendix I) and stored at 4°C. Commercially prepared rabbit anti-sheep erythrocytic serum (Wellcome Reagents Ltd.) was used to sensitize sheep erythrocytes.

Erythrocytes. Sheep erythrocytes were collected from the jugular vein into bottles containing Alsever's solution (Appendix I) to give one part of blood to one part of solution. The cells were washed twice in Alsever's solution and stored at $4^{\circ}C$ until required. Immediately before use an aliquot of stock erythrocytes was transferred to a centrifuge tube and veronal buffer added to wash the cells by gentle pipetting. They were washed until they were clear of any haemolysed cells and sufficient VB added to make a four percent suspension.

Titration of complement and haemolytic system. The haemolytic system and complement were titrated in respect to each other in a chequer-board (Hoskins, 1967) after adding equal volumes of a four percent suspension of sheep erythrocytes and consecutive dilutions of anti-sheep erythrocytic serum. For the CF test two units of complement and two optimum haemolytic doses were used, one unit of complement being the highest dilution of complement which caused complete, 100 percent, haemolysis of two percent sheep erythrocytes sensitised with optimum amounts of haemolysin.

Antigen. Antigens were prepared from leucocytes of infected sheep as described by Snodgrass and Ramachandran (1971) with minor modifications. Briefly, sheep were bled at the peak period of parasitaemia into bottles containing heparin to give a concentration of 20 IU of heparin per ml. The erythrocytes were lysed with 0.83 percent ammonium chloride solution and the remaining cells pelleted by centrifugation at 2000 g for 15 minutes. The cells were washed three times with PBS at pH 7.2 and resuspended in VB to give at

least 10^7 infected cells per ml. They were disintegrated with ultrasonic vibration (MSE Ltd.) at optimum amplitude for 90 seconds, by alternate freezing and thawing or by passing them through an X-press (Life Science Lab. Ltd.). The X-press works on the principle that by forcing cells frozen at $-25^{\circ}C$ through a hole much smaller than the diameter of the cylinder in which the cells are contained but larger than the size of individual cells, a satisfactory disintegration of the cells can be obtained (Edebo, 1960). A frozen suspension of infected leucocytes was disrupted after being forced through the X-press cylinder by an hydraulic pressure of four tons. The antigens were stored in screw-capped glass bottles at $-20^{\circ}C$ or $-114^{\circ}C$ until use.

Sera. Sera were collected from 25 sheep before inoculation with C. phagocytophila and every week thereafter for eight weeks and then every month for variable periods. Some of the sheep were used for the experiments in Chapters Seven and Eight. Sheep sera showed strong anti-complementary activitites and this was reduced by treating them with guinea pig complement before use.

Titration of antigens and sera. When the titres of antigen and sera were unknown they were titrated against each other in a chequer-board. All tests were carried out in microtitre plates (Sterilin Ltd.) and all reagents delivered with micropipettes calibrated to give 0.25 ml.

Interpretation of results. All tests were declared invalid if serum and antigen controls were not haemolysed. The interpretation of reactions between no haemolysis to complete haemolysis

was based on scoring them as follows:

4 = no haemolysis

3 = 25 percent haemolysis

2 = 50 percent haemolysis

1 = 75 percent haemolysis

0 = 100 percent haemolysis

Wells scoring four or three were regarded as positive, i.e. complement fixation was considered to have occurred. The serum titre was defined as the highest dilution of serum showing positive reaction in the presence of two units of antigen and two units of complement. Once standard positive serum was established new antigen preparations were titrated against a 1/8 dilution of standard positive serum. The titre of antigen was expressed as the highest dilution of antigen which fixed complement in the presence of standard immune serum. For the test proper two units of antigen were used.

Immunofluorescent Test

Preparations of antigen. Antigens were prepared by three different methods.

Buffy coat. The buffy coat of infected blood was separated by centrifugation at 1500 *g* for ten minutes, washed three times with PBS and thick smears made on microscopic slides.

Buffy coat treated with 0.83 percent ammonium chloride. The buffy coat was separated and the red blood cells lysed with 0.83 percent ammonium chloride solution, washed with PBS and thick smears prepared.

Attachment of leucocytes onto ooverslips and multispot slides. The method was based on the properties of granulocytes and monocytes to attach to glass surfaces (Koske, 1976). Buffy coat preparations from infected blood were treated with 0.83 percent ammonium chloride solution to lyse red blood cells, washed with PBS and resuspended in RPMI 1640 with HEPES buffer and 20 to 50 percent autologous plasma, as described in Chapter Three, or in 0.5 percent bovine serum albumin to an approximate concentration of 10^6 cells per ml. The cells were distributed into Leighton tubes (Gallenkamp Ltd.), containing micro-coverslips, or multispot slides (C.A. Hendley and Co.) and incubated at $37^{\circ}C$ for two to four hours to allow the granulocytes and monocytes to attach to glass surfaces. The unattached cells were washed off by rinsing the coverslips and slides with PBS. All slides and coverslips were fixed in acetone for ten minutes, wrapped in tissue paper and aluminium foil and stored at $-20^{\circ}C$ until required.

Preparation of anti-TBF globulins. IgM and IgG fractions of immune sera were prepared as described in Chapter Eight. Those fractions which had high CF antibody titres were pooled and the protein content estimated by the biuret method (Lowry, Rosebrough, Farr and Randall, 1951). The globulins were diluted in a carbonate-bicarbonate buffer pH 9.0 (Schneider, 1973) and to each mg of protein 0.02 mg of fluorescein isothiocyanata added. The mixture was stirred magnetically at $4^{\circ}C$ overnight. The fluorescein not bound to protein was removed by gel-filtration on a Sephadex column with Sephadex G-25 (Sephadex Fine Chemicals, Uppsala) and the conjugate eluted with PBS at pH 7.6. The conjugate was adsorbed

with acetone-dried calf liver and fresh normal sheep leucocytes and stored at -20°C until use.

Anti-sheep globulins. Fluorescein conjugated anti-sheep globulin was commercially supplied (Grand Island Biological Company).

Staining procedure. Slides and coverslips were withdrawn from the deep-freeze and left at 4°C for 30 minutes and at room temperature for a further 30 minutes before use. For the direct fluorescent antibody test two-fold dilutions of anti-TBF conjugate were prepared and allowed to react with antigen at room temperature or at 37°C for 30 minutes, one hour or two hours. The preparations were then washed with PBS for 10 minutes three times, washed in distilled water for three minutes and counterstained with a 10^{-5} dilution of Evans' blue, if necessary. After washing off the counterstain with distilled water the slides or coverslips were mounted with phosphate-buffered glycerol at pH 7.2 (Difco Lab., Detroit).

For the indirect immunofluorescent test dilutions of sera were allowed to react with antigen preparations at 37°C or room temperature for 30 minutes, one hour or two hours and then washed with PBS at least three times and dried. Then the anti-sheep conjugate was allowed to react at 37°C for 30 minutes and washed as described for the direct immunofluorescent test.

Microscopy. The slides and coverslips were observed with a Nikon fluorescence microscope with Mercury vapour for the presence of fluorescent particles with morphological similarities to the various forms of C. phagocytophila.

Enzyme-Linked Immunosorbent Assay (ELISA)

Preparation of antigens. The technique for preparing antigens was similar to that described by Halle, Dasch and Weiss (1977) for preparing rickettsial antigens. Briefly, antigens were first processed as described for the CFT. Then the disintegrated infected cells were mixed with three volumes of acetone and left overnight at room temperature. They were centrifuged at 2000 g for 20 minutes and the insoluble pellet resuspended in acetone. The process was repeated twice. The powder was tested for CF activity before further extraction with ether and use for ELISA.

Pig IgG and IgM linked with horse radish peroxidase obtained from pigs immunized against sheep globulins were commercially supplied (Eivai Bios Laboratories Ltd.).

Micro-ELISA technique. Antigen dilutions in 0.1M sodium bicarbonate buffer (pH 9.6) were coated onto flat wells of disposable polystyrene plates (Sterilin Ltd.) in 0.3 ml quantities by incubating them at 37°C for one hour and then leaving them at 4°C overnight. A series of antigen dilutions were set up to determine the optimum antigen dilution. Materials prepared from uninfected sheep leucocytes were used as controls. Fluids were aspirated and all wells amply washed three times with normal saline containing 0.05 percent (w/v) Tween 20 (Sigma). Serum dilutions were prepared in PBS with 0.05 percent Tween 20 and 0.3 ml added to the appropriate wells. The plates were incubated for two hours at room temperature and then washed with normal saline and allowed to air-dry. Then the conjugate in PBS with Tween 20 was added,

having been titrated previously, and left in a dark place at room temperature overnight. The plates were once more amply washed three times with normal saline. The enzyme substrate, ortho-phenylene diamine (Sigma), was prepared immediately before use by dissolving 35 mg of substrate in 100 ml citric acid/phosphate buffer, pH 6.0 and 0.05 percent hydrogen peroxide ("Analar", British Drug House). Then 0.3 ml of the substrate was added into each well and the plate left in the dark (Brandt, 1980). The colour changes were regularly checked and as soon as the positive controls had taken an orange colour, the reaction was stopped by adding 50 µl of 1M sulphuric acid (Aristar, BDH). The reaction's absorbance was assessed by a spectrophotometer (SPG-200 Pye Unicam) at 455 nm filter, using a glass cuvette of one cm light path. Absorbance was set at zero using negative controls.

Indirect Haemagglutination Test (IHT)

Sensitization of erythrocytes. Sheep erythrocytes were collected in Alsever's solution as described earlier and stored at $4^{\circ}C$ until required. They were washed with normal saline three times and then with PBS at pH 7.2. They were coated with antigen with either gluteraldehyde or tannic acid as coupling agents. In the former case eight ml of a 2.5 percent erythrocyte suspension was mixed with one ml of 2.5 percent gluteraldehyde in PBS and an equal amount of high-titred C. phagocytophila antigen, previously titrated with positive serum by the complement fixation test, added. Sensitization was allowed to proceed at $37^{\circ}C$ with gentle magnetic stirring for one hour by which time the cells had a

brownish appearance. The cells were washed three times with PBS containing 0.2 percent bovine albumin as a stabiliser and 0.02 percent sodium azide as a preservative. Control cells were similarly treated with gluteraldehyde and leucocyte extract from uninfected sheep. In the latter method the cells were washed with PBS at pH 7.2 before being tanned. Briefly, a 1.5 percent suspension of cells was prepared and immediately treated with a 1:40000 dilution of tannic acid or a ten percent suspension was treated with a 1:5000 dilution of tannic acid (Herbert, 1974). The tannic acid was adjusted to pH 7.2 with 1M sodium carbonate. The tanning took place at 37°C in ten minutes. After tanning, the cells were washed with PBS twice before coating them with antigen as described earlier.

The test. The tests were carried out in V-shaped 96-well microplates (Sterilin Ltd.). Test sera were first inactivated at 56°C for 30 minutes and then adsorbed with cells coated with extracts of leucocytes from uninfected sheep. A series of two-fold dilutions were then prepared in microplates and an equal amount of 0.5 percent coated cells added. They were allowed to react at 37°C for ten minutes under continuous magnetic stirring and left at 4°C overnight and results visually interpreted. The antibody titre was taken as the highest dilution of serum causing over 50 percent agglutination.

RESULTS

Complement Fixation Test

Complement fixing antibodies were detected in sheep sera two weeks after experimental infection with C. phagocytophila. Antigens were not consistently produced from infected blood, but the problem was resolved by improving the antigen yields as described later in Chapter Six. Infected cells were effectively disrupted by ultrasonic vibration for 90 seconds and passing them through an X-press at least four times. Alternate freezing and thawing was not effective. Although the X-press was as effective as the ultrasonic treatment it was time consuming and some antigens were lost in the process of cooling and pressing. The antigens were found resistant to heating at 56°C and 60°C for up to 30 minutes. The antigens did not lose their activities after being stored at -20°C or -114°C for over two years. A limited attempt to chemically characterise the antigen was carried out. High-titred antigens were treated with trypsin, phosphatide acylhydrolase and potassium periodate as described by Kobayashi, Nagai and Tachibana (1969). There was no reduction in the titre of the antigens after treatment with trypsin but two-fold reductions in the antigen titres were recorded when the antigens were treated with either potassium periodate or phosphatase.

The antigen did not fix complement when allowed to react with sera of sheep with known high titres of antibodies against Chlamydia. The sera were kindly provided by Dr. I.D. Aitken of the Moredun Institute, Edinburgh.

Sera from 25 sheep were studied for long periods after infection. No antibodies were detected in all but three sheep from sera collected before inoculation (Figure 11 and Table 11). One week after infection antibodies were detected in six sheep but two weeks after infection CF antibodies were present in the sera of all sheep but one. The highest CF titres were detected three weeks after infection. The CF antibody titre remained at high levels for the first eight weeks, gradually declining thereafter. However, CF antibodies were detected for more than one year.

Immunofluorescent Test

Conjugates prepared from globulins of immune sheep produced specific fluorescence in the cytoplasms of infected cells. Single as well as clusters of particles were observed and they resembled those seen after staining infected cells with Giemsa (Figure 12). The good antigen preparations were those prepared from buffy coat cells without lysis of red blood cells and with little washing. Those which were washed repeatedly appeared to lose their fluorescence as did those which were treated with 0.83 percent ammonium chloride. Granulocytes and monocytes, including infected cells attached to glass surfaces. Attachment to glass surfaces increased the percentage of granulocytes significantly ($t_{10} = 10.9$, $p < 0.001$; Appendix Table 11) but the increased number of granulocytes per microscopic field did not offer any advantages because there was a reduction in the proportion of infected cells after attachment ($t_{10} = 5.03$, $p < 0.001$; Appendix Table 11) and the attached cells

appeared less spread making differentiation difficult. An incubation of 30 minutes at 37°C or one hour at room temperature was found sufficient for the reaction. The specificity of the reaction was tested by allowing antigens to react with unconjugated immune serum prior to the addition of anti-TBF conjugate. When low dilutions of immune serum were added the fluorescence was completely masked but high dilutions did not mask fluorescence.

Excessive non-specific fluorescence rendered the indirect immunofluorescent test impossible.

ELISA

Antigen-antibody reactions were bedeviled by excessive non-specific reactions even with control antigens prepared from extracts of normal sheep leucocytes. The technique was, therefore, discontinued.

Indirect Haemagglutination Test

The results with the IHT were not consistent, the main problem being non-specific agglutination. The tannic acid was found to be a better coupling agent than gluteraldehyde but because of the limitations of antigen extensive comparisons were not attempted and coating with tannic acid as a coupling agent was adopted. Antigens with very high titres for the CF test did not always effectively coat the cells. Because of the high failure rate only few successful tests were carried out. Eighteen sera were tested with IHT and CFT and the CF and IH titres compared. These limited results suggested that the IH titres were significantly lower than the CF titres ($t_{17} = 6.43$, $p < 0.001$; Figure 13 and Appendix Table 12).

Figure 11 Mean CF antibody titres of sera sequentially collected from sheep infected with *C. phagocytophila*

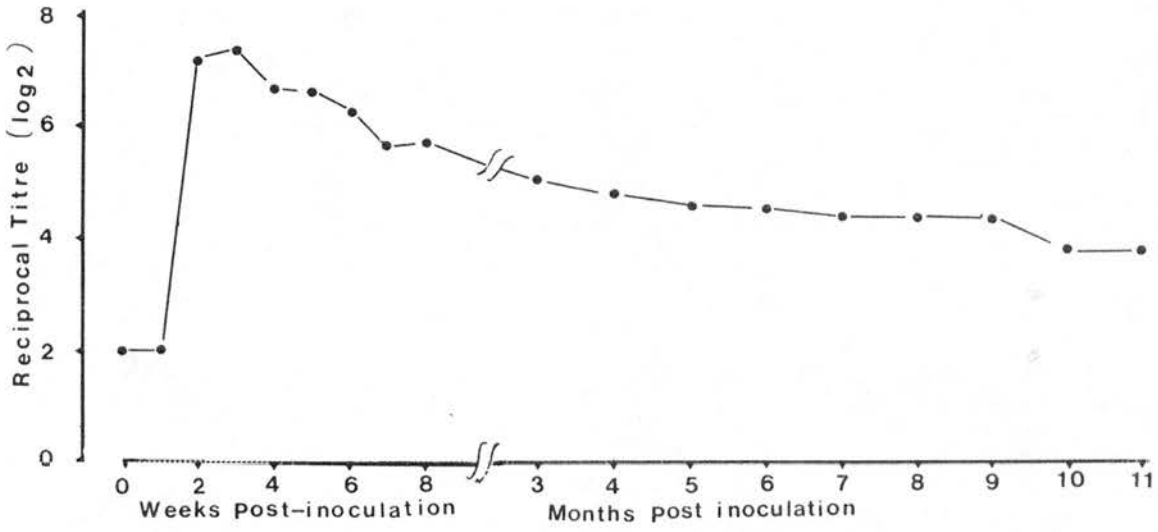

Reciprocal Titre (log2)
8
6
4
2
0
0
2
4
6
8
Weeks Post-inoculation
3
4
5
6
7
8
9
10
11
Months post inoculation

Figure 12 Direct immunofluorescence

An infected leucocyte with a cytoplasmic inclusion containing fluorescing clusters after treatment with anti-TBF conjugate x 1800

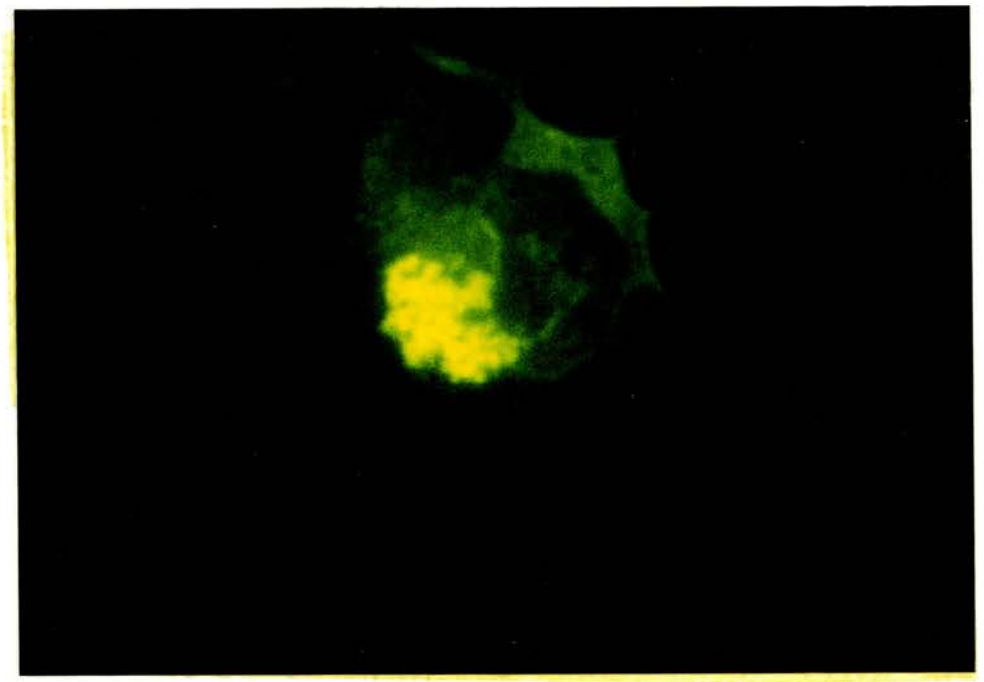


Figure 13 Comparison of the CF and IH antibody titres of sera from infected sheep

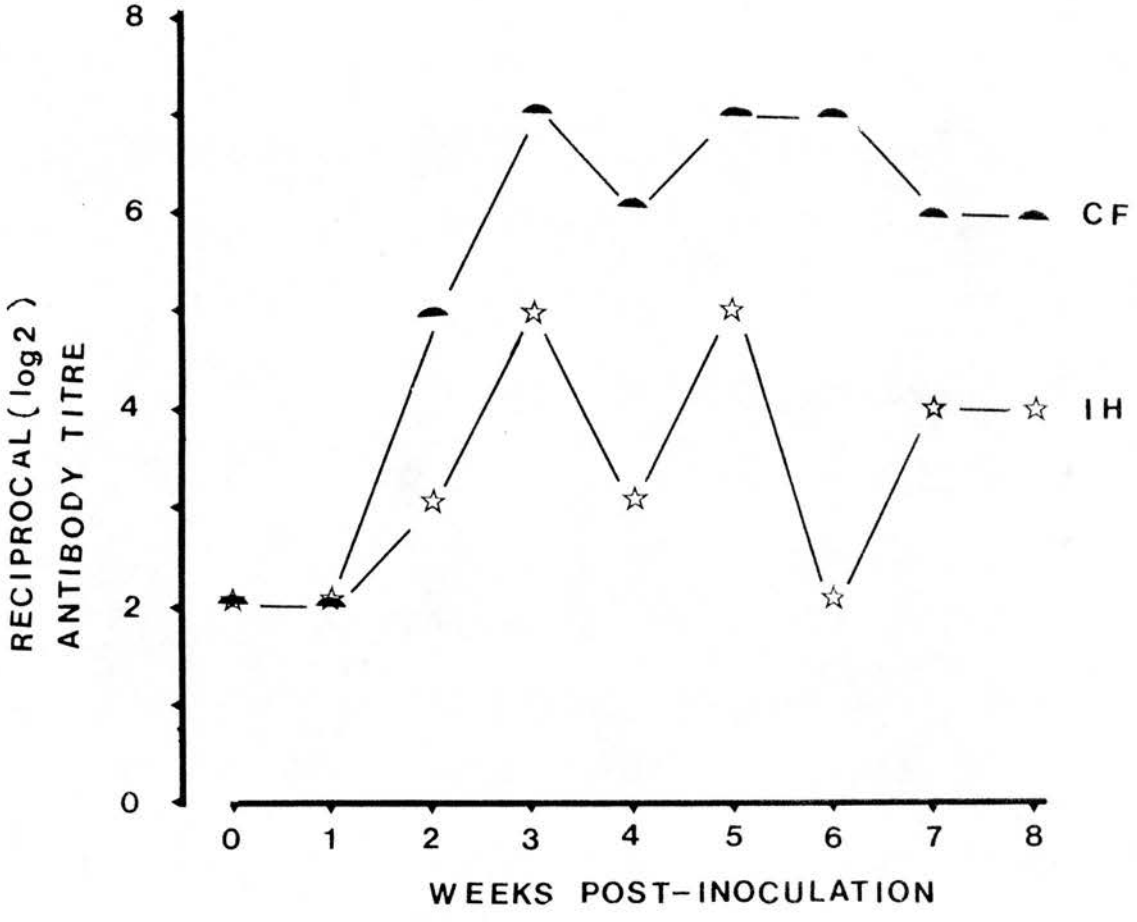

8
6
4
2
0
RECIPROCAL (log2)
ANTIBODY TITRE
CF
IH
0
1
2
3
4
5
6
7
8
WEEKS POST-INOCULATION

Table 11 Reciprocal CF antibody titres ($\log_2$) of sequential sera from sheep infected with TBF

Weeks post-inoculation	Number of sheep	Number of positive sera	Mean	Standard deviation
pre inoculation	25	3	3.33[a]	0.58
1	25	6	3.67[b]	1.21
2	25	24	7.16	1.85
3	25	25	7.32	1.55
4	25	25	6.64	1.55
5	25	25	6.56	1.23
6	25	25	6.24	0.97
7	25	25	5.60	1.19
8	25	25	5.64	1.50
12	25	25	5.04	1.34
16	23	23	4.83	1.43
20	17	17	4.63	0.96
24	17	17	4.59	1.12
28	18	18	4.56	1.14
32	17	17	4.47	1.23
36	15	15	4.53	1.19
40	15	15	3.73	0.80
44	15	15	3.87	0.64
48	14	14	4.00	0.55
52	14	14	4.00	0.96
56	13	13	3.38	0.77
60	10	10	3.20	0.63
64	6	6	3.50	0.55
68	3	3	3.67	0.58

[a] mean titre of three positive sera

[b] mean titres of six positive sera

DISCUSSION

The CF test was found reliable and reproducible. The antigens were prepared either with ultrasonication or with an X-press but the former was preferred for its speed and because of the loss of antigen in the latter. The antigen was found to be more stable than was originally reported by Snodgrass (1974). Heating at 56°C or 60°C for 30 minutes did not affect antigenic activity. I found no reduction of antigen titre after prolonged storage at -114°C or even at -20°C. The limited chemical tests suggested that the antigens might be lipo-polysaccharides but chemical analysis of purified organisms will be needed to confirm this. The antigens did not fix complement when allowed to react with antibodies against Chlamydia. The last finding supports Foggie's (1962^b) earlier report that Cytoecetes phagocytophila had no antigenic relationship with the Chlamydia.

The direct fluorescent antibody technique was also found to be reliable. The limiting factors were the availability of high-titred anti-TBF conjugate and well prepared antigens; for example, I prepared some conjugate from sheep immune to TBF before the CF test was developed and the results were discouraging. When the CF test was developed it was found that the serum used had a reciprocal titre of 2^3. After the development of the CFT it became feasible to choose high titred sera. When conjugates were prepared from sera with reciprocal titres of more than 2^7 the results were good. Of the three methods used to prepare antigen the one using thick buffy coat smears with little or no washing

was best. This was similar to the method originally described by Tuomi (1967[d]). However, because of the presence of plasma, this method was not ideal for the indirect immunofluorescent test. Allowing cells to attach to coverslips after washing them with PBS and resuspending them in RPMI 1640 medium with 0.5 percent bovine serum albumin eliminated the problem of non-specific fluorescence but there was no specific fluorescence. This suggested that either the anti-sheep globulin was very weak or the preparation of the antigen was not ideal; the antigen determinants were probably affected by washing. The indirect fluorescent test merits further investigation.

The results with ELISA were discouraging. The test is based on the use of purified and soluble antigens. Some workers have used acetone-ether extraction as a means of solubilizing rickettsial antigens (Halle et al., 1977) but there was no evidence that this method was successful in the extraction of C. phagocytophila antigens. Using the CF test I found that after extraction with acetone and ether the antigenic activity still remained in the particulate material.

Since its introduction by Engvall and Perlmann (1972), the enzyme-linked immunosorbent assay (ELISA) has found wide use for immunological studies. The test is reported to be sensitive but requires purified and soluble antigens. Until such time when pure C. phagocytophila can be obtained and solubilized we cannot make use of this sensitive test.

The results with the IHT were inconsistent and in the limited successful tests the IH titres were lower than the CF titres. Of

all the tests tried in this study the CF test and the direct fluorescent test appeared to be more reproducible. The direct fluorescent test will be an important tool in studies concerning the in vitro propagation of the organism and in studying the mechanisms by which the organism multiplies in the host before it appears in the blood and the ways it survives in an apparently immune host. The CF test has been found superior to the other tests in its reproducibility and sensitivity provided high-titred antigens are used. The problem regarding antigen yields is dealt with in the next Chapter.

CHAPTER SIX

IMPROVEMENT OF ANTIGEN YIELDS

INTRODUCTION

The complement fixation test has been found to be reliable and reproducible but low antigen yields have limited its wide application (Snodgrass, 1974; Chapter Five). Snodgrass and Ramachandran (1971) used the leucocytes of infected sheep as sources of antigen. I also used the infected leucocytes but the antigen yields were very low. The low yields of antigen can be attributed to the reduction of total leucocytes which accompanies the disease (Taylor et al., 1941; Chapter Two). Although a greater proportion of the granulocytes are infected during the peak period of febrile reaction the transient period of neutrophilia is quickly followed by a severe reduction of neutrophils. Attempts were made, therefore, to increase the number of circulating granulocytes with the ultimate aim of increasing the total number of infected cells. It has been long established that certain corticosteroids induce a transient but marked granulocytosis (Mishler, 1977). Experiments were designed first to study the effects of corticosteroids on the granulocytes of sheep infected with C. phagocytophila and the possible increase in the number of infected cells. Secondly antigens were prepared from leucocytes of sheep infected with TBF and subsequently treated with the corticosteroid. Other experiments were designed to study the effect of separating the granulocytes from the other components of blood on the antigen titre. A third attempt was made to increase the antigen yield by increasing the number of infected cells and the number of organisms per infected cell by culturing infected blood as described in Chapters Three and Four.

MATERIALS AND METHODS

Treatment with Corticosteroid

Animals. Adult sheep varying in age, breed and sex were obtained from tick-free areas. They were inoculated with a stabilate of the OS strain as described in Chapter Two.

Eleven infected sheep were injected IV with one mg of the corticosteroid betamethasone sodium phosphate injection BP per Kg body weight at the second day of parasitaemia. Nine infected sheep were injected with 0.5 ml of normal saline per Kg body weight as controls. All animals were bled into tubes containing dipoatassium ethylendiamine tetra-acetic acid (EDTA) immediately before infection and every hour thereafter for eight hours. They were also bled 24 and 48 hours post-injection.

The total leucocytes were counted electronically (Coulter Electronics Ltd., England). The absolute numbers of the granulocytes, lymphocytes and monocytes were calculated from the total leucocytes. Parasitised granulocytes were expressed as percentages of leucocytes or as percentages of granulocytes. The absolute numbers of infected cells were derived from the total leucocytes.

Culture of Infected Blood

Blood from infected sheep was collected in sterile containers with heparin to give a concentration of 20 IU per ml. The blood was mixed with three volumes of medium 199 containing 25 mM HEPES buffer and 1 mM glutamine. Ten percent foetal calf serum or lamb serum and 100 IU of penicillin per ml were incorporated immediately before use. The mixture was left at 37°C under continuous magnetic

stirring for up to 24 hours. Samples were taken after four, eight and 24 hours and the infection rate of granulocytes recorded. The whole blood mixtures were used as sources of antigen.

Separation of Granulocytes

The granulocytes were separated from other components of heparinised peripheral blood by density gradient centrifugation. Lymphoprep (Nyegaard and Co., A.S., Oslo) was used to give a final density gradient of 1.077 g per ml at 20°C. To reduce the quantity of starting material blood collected at the peak period of parasitaemia was centrifuged in siliconised containers at 1500 g for 10 minutes. The buffy coat was separated and resuspended in PBS. The buffy coat suspensions was then gently layered onto lymphoprep in siliconised polycarbonate test tubes to make a ratio of 4:3. After centrifugation at 400 g for 30 minutes the granulocytes and the erythrocytes were pelleted and the mononuclear cells formed a separate layer at the interface between the lymphoprep and the supernatant. The supernatant and the mononuclear cell layers were discarded and the pellet harvested to be used as sources of antigen.

Sources of Antigen

Whole infected blood, infected blood collected after treatment of infected sheep with corticosteroid, whole infected blood cultured for 24 hours and infected granulocytes separated by gradient centrifugation were used as sources of antigen.

Preparation of Antigen

The red blood cells in the antigen sources were lysed with 0.83 percent ammonium chloride solution and the remaining cells washed three times in PBS. The cells were suspended in veronal buffer to give about 10^7 infected cells per ml. They were disintegrated in aliquots of two ml with an ultrasonicator (MSE Ltd.) for 90 seconds at optimum amplitude.

Sera

Sera collected two to three weeks after experimental infection with C. phagocytophila (Snodgrass and Ramachandran, 1971; Chapter Five) were used to titrate antigens. Sera from uninfected sheep were used as controls. All sera were treated with complement to eliminate anti-complementary activities.

Complement Fixation Test

The CF test was carried out as described in Chapter Five.

RESULTS

Response to Treatment with Corticosteroids

When infected sheep were injected with the corticosteroid significant clinical and haematological changes occurred within on hour.

Rectal temperature. A dramatic reduction in the rectal temperature occurred as early as one hour after treatment, the mean reduction being $0.75 \pm 0.48^{\circ}C$ (Figure 14). The temperature continued to fall, reaching a maximal reduction of $2.20 \pm 0.29^{\circ}C$

after five hours. After 24 hours the temperature had risen but was still significantly lower than the pre-infection temperature ($t_{10} = 4.67$, $p < 0.001$; Tables 12 and 13, Appendix Tables 13 and 14).

Total leucocytes. An increase in the total leucocytes was observed one hour after the injection (Figure 14). The increase of $2.46 \times 10^9 \pm 1.42 \times 10^9$ cells per litre was statistically very significant ($t_{10} = 5.72$, $p < 0.001$; Tables 14 and 15, Appendix Tables 15 and 16). The total leucocytes remained at high levels for at least eight hours, returning to pre-treatment levels after 24 hours.

Granulocytes. Very significant increases in the number of granulocytes were observed within an hour of infection ($t_{10} = 5.69$, $p < 0.001$). The granulocytes continued to rise, reaching a maximal increase of $4.13 \times 10^9 \pm 1.75 \times 10^9$ cells per litre after four hours. The granulocyte numbers fell after eight hours (Figure 15) but were still significantly higher than pre-infection levels ($t_{10} = 5.17$, $p < 0.001$; Tables 16 and 17, Appendix Tables 17 and 18). An increase in the number of band or immature granulocytes was also observed.

Lymphocytes. A slight reduction in the number of circulating lymphocytes was observed (Figure 15, Tables 18 and 19, Appendix Tables 19 and 20).

Infected cells. The absolute number of infected cells was significantly increased as early as one hour after injection (Figure 15, Tables 20 and 21, Appendix Tables 21 and 22) but the

percentage of infected granulocytes was significantly reduced following injection of betamethasone (Figure 16, Tables 22 and 23, Appendix Tables 23 and 24). The highest increase in the total number of infected cells was recorded six hours after injection, the increase being $1.16 \times 10^9 \pm 0.6 \times 10^9$ infected cells per litre.

Comparison of Antigen Yields

Antigen titres of infected blood collected after treatment with corticosteroid. Studies were carried out to compare the antigen yields of infected blood collected at the peak period of parasitaemia with that which was collected six hours after injection with corticosteroid. For this purpose eight infected sheep were used. At the peak period of parasitaemia they were bled 100 ml of blood in heparinised containers. Subsequently they were injected with corticosteroid as described above and six hours later they were bled 100 ml. Antigens were prepared and the titres of the antigens prepared from infected blood collected before treatment and those prepared from blood collected six hours after treatment compared. Antigen prepared from the latter were significantly higher than those prepared from blood collected before treatment ($t_8 = 3.94$, $p < 0.01$, Appendix Table 25).

Antigen titres of cultured infected blood. When infected blood was cultured with HEPES-buffered medium 199 significant increases in the number of infected cells occurred within 24 hours (Chapter Three). Studies were carried out to compare the antigen yields of infected blood cultured for four, eight and 24 hours

with infected blood processed immediately after collection. Infected blood was obtained at the peak period of parasitaemia and divided into two groups. One groups was processed for antigen immediately while the other groups were processed after four, eight and 24 hours of culture. The titres of the antigens processed after culture were higher than those processed before culture as early as four hours after culture but the increases were significant after eight hours and 24 hours (Table 24, Appendix Table 26).

Antigen yields of granulocytes separated by density gradient centrifugation. A comparison of the antigen yields of infected granulocytes separated by density gradient centrifugation and of antigen prepared from the buffy coat without separation of granulocytes were made. For this purpose nine infected sheep were bled into heparinised containers and the buffy coat processed as described earlier. The buffy coat was then divided into two equal samples. Antigens were prepared from one sample after separation of granulocytes while from the other one the antigen was prepared without separating the granulocytes. When granulocytes of infected sheep were separated by denisty gradient centrifugation, a slight, but statistically insignificant, increase ($6_8 = 1.79$, $p > 0.10$) in the titre of the antigens was observed. The recovery rate of the granulocytes was 88.67 ± 9.49 percent but the loss of infected neutrophils was statistically significant ($t_8 = 4.64$, $p < 0.010$; Appendix Table 27)

Figure 14 Mean total leucocytes and rectal temperature of TBF-infected sheep injected with beta-methasone

A - total leucocytes

B - rectal temperature

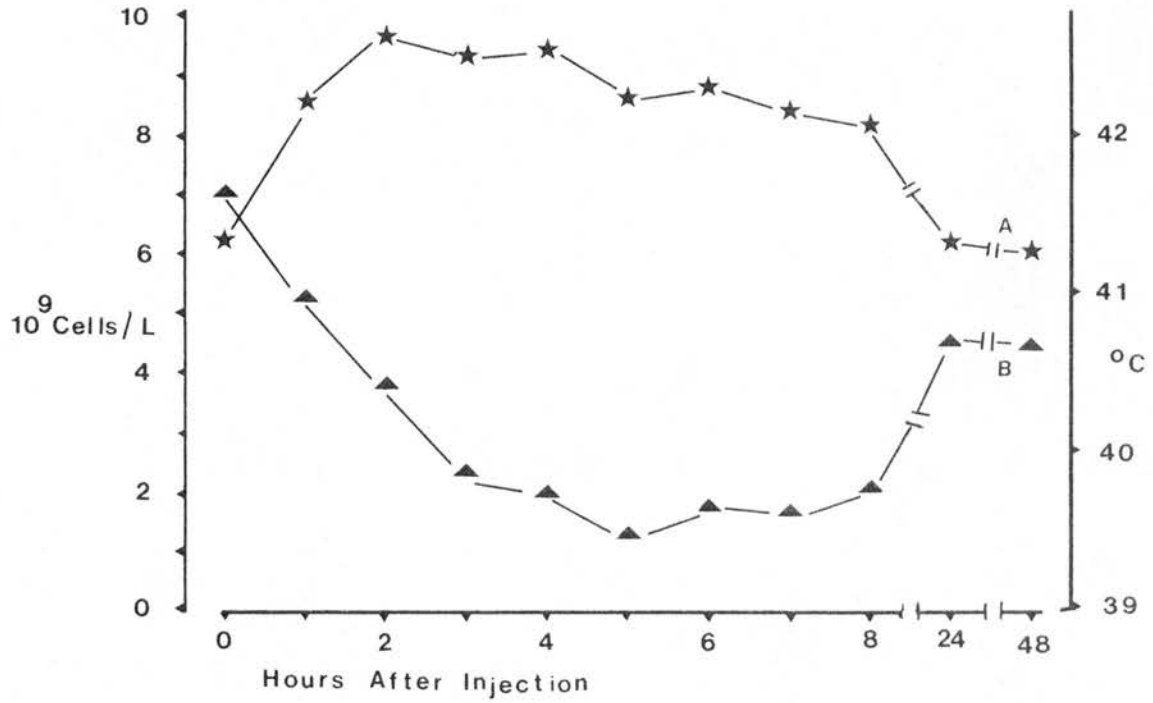

10
8
6
4
2
0
10^9 Cells / L
42
41
40
39
°C
A
B
0
2
4
6
8
24
48
Hours After Injection

Figure 15 Mean total granulocytes, lymphocytes and infected cells of TBF-infected sheep injected with betamethasone

A - total infected cells

B - total lymphocytes

C - total granulocytes

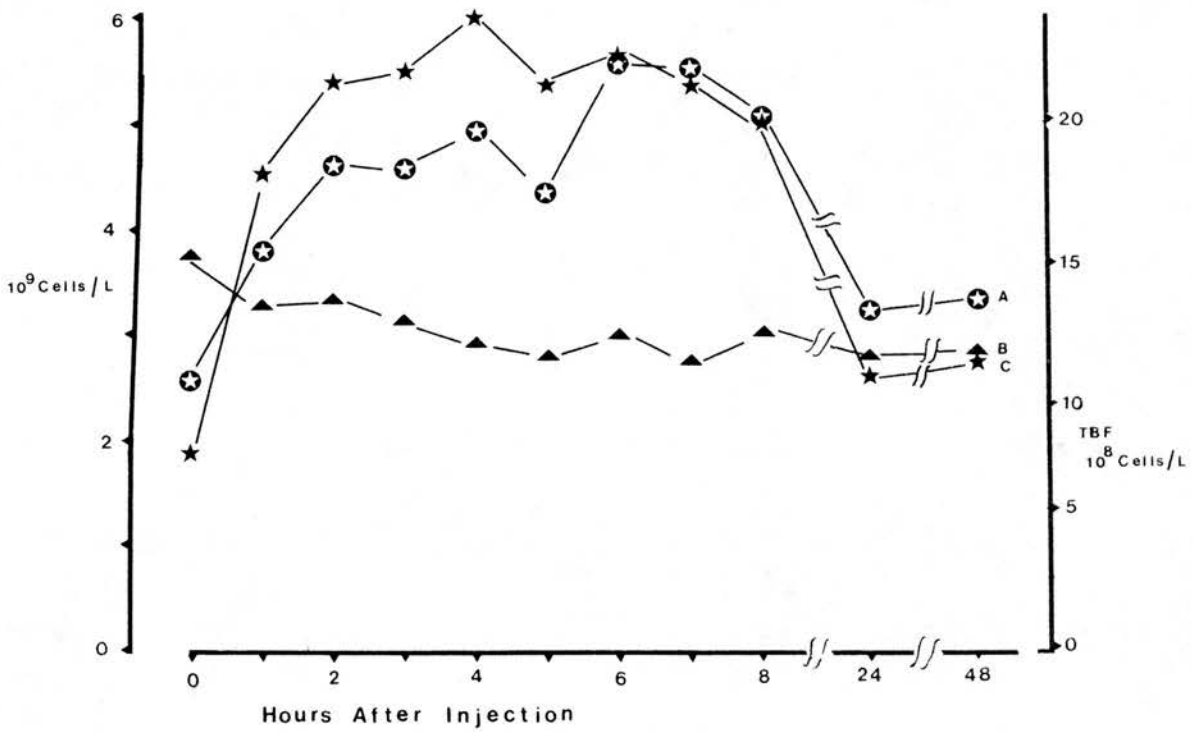

6
4
2
0
10^9 Cells / L
20
15
10
5
0
TBF
10^8 Cells/L
A
B
C
0
2
4
6
8
24
48
Hours After Injection

Figure 16 Mean total granulocytes and percentage of infected granulocytes after injection with betamethasone

A - Total granulocytes

B - Percentage of infected granulocytes

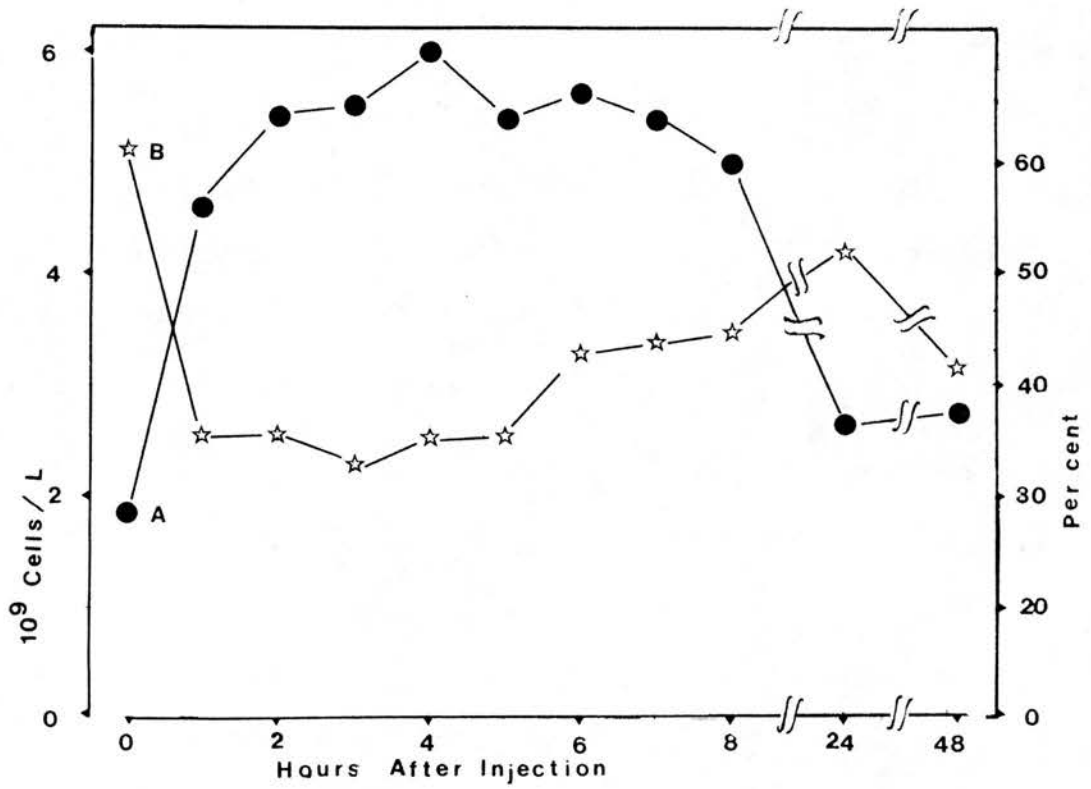

6
4
2
0
10^9 Cells / L
B
A
60
50
40
30
20
0
Per cent
0
2
4
6
8
24
48
Hours After Injection

Table 12 Deviations of rectal temperatures of infected sheep after injection with betamethasone (Hour 0 - Hour X)

Hours post-injection	Degrees of freedom	Mean difference (°C)	e	t
1	10	-0.75	0.14	5.36***
2	10	-1.35	0.09	15.00***
3	10	-1.73	0.10	17.30***
4	10	-1.97	0.11	17.91***
5	10	-2.20	0.09	24.44***
6	10	-2.13	0.11	19.36***
7	10	-2.01	0.13	15.46***
8	10	-1.94	0.12	16.17***
24	10	-0.98	0.21	4.67***
48	7	-0.98	0.18	5.44***

*** $P < 0.001$

Table 13 Deviations of rectal temperatures of infected sheep after injection with normal saline (Hour 0 - Hour X)

Hours after injection	Mean difference (°C)	e	$t_{(8)}$
1	-0.08	0.12	0.67
2	0.04	0.13	0.31
3	0.06	0.14	0.43
4	0.10	0.14	0.71
5	0.11	0.17	0.65
6	0.01	0.18	0.66
7	-0.03	0.18	0.17
8	0.01	0.19	0.05
24	0.33	0.12	2.75*
48	0.64	0.31	2.06

* $P < 0.050$

Table 14 Deviations of total leucocytes of infected sheep after injection with betamethasone

Hours post-injection	Degrees of freedom	Mean difference (10^9/l)	e	t
1	10	2.46	0.43	5.72***
2	10	3.46	0.60	5.77***
3	10	3.41	0.58	5.88***
4	10	3.31	0.53	6.25***
5	10	2.58	0.41	6.29***
6	10	2.77	0.53	5.23***
7	10	2.42	0.51	4.75***
8	10	2.27	0.62	3.66**
24	10	0.06	0.49	0.12
48	7	-0.06	0.79	0.08

*** $P < 0.001$; ** $P < 0.010$

Table 15 Deviations of total leucocytes of infected sheep after injection with normal saline (Hour 0 - Hour X)

Hours post-injection	Mean difference (10^9/l)	e	$t_{(8)}$
1	0.08	0.16	0.50
2	0.22	0.37	0.59
3	0.41	0.38	1.08
4	0.80	0.41	1.95
5	0.44	0.49	0.90
6	0.21	0.25	0.84
7	0.34	0.31	1.10
8	0.25	0.37	0.68
24	0.81	0.63	1.29
48	1.06	0.67	1.58

Table 16 Deviations of total PMN of infected sheep after injection with betamethasone (Hour 0 - Hour X)

Hours post-injection	Degrees of freedom	Mean difference (10^9/l)	e	t
1	10	2.73	0.48	5.69***
2	10	3.62	0.55	6.58***
3	10	3.69	0.51	7.24***
4	10	4.13	0.53	7.79***
5	10	3.51	0.51	6.88***
6	10	3.78	0.56	6.75***
7	10	3.45	0.54	6.39***
8	10	3.10	0.60	5.17***
24	10	0.78	0.30	2.60*
48	7	1.11	0.71	1.56

*** $P < 0.001$; * $P < 0.050$

Table 17 Deviations of total PMN of infected sheep after injection with normal saline

Hours post-injection	Mean difference (10^9/l)	e	$t_{(9)}$
1	0.26	0.11	2.36*
2	0.02	0.21	0.10
3	0.19	0.21	0.90
4	0.37	0.26	1.42
5	0.24	0.25	0.96
6	0.24	0.13	1.85
7	0.28	0.20	1.40
8	0.26	0.16	1.63
24	0.29	0.40	0.73
48	0.31	0.61	0.51

*$P < 0.050$

Table 18 Deviations of total lymphocytes of infected sheep after injection with betamethasone (Hour 0 - Hour X)

Hours post-injection	Degrees of freedom	Mean difference (10^9/l)	e	t
1	10	-0.23	0.32	0.72
2	10	-0.43	0.27	1.59
3	10	-0.33	0.32	1.03
4	10	-0.85	0.31	2.74*
5	10	-0.95	0.21	4.52**
6	10	-0.75	0.36	2.08
7	10	1.04	0.25	4.16**
8	10	-0.96	0.23	4.17**
24	10	-0.88	0.35	2.51*
48	7	0.98	0.28	3.50**

** $P < 0.010$; * $P < 0.050$

Table 19 Deviations of total lymphocytes of infected sheep after injection with normal saline (Hour 0 - Hour X)

Hours post-injection	Mean difference (10^9/l)	e	$t_{(8)}$
1	0.41	0.17	2.41*
2	0.32	0.24	1.33
3	0.27	0.27	1.00
4	0.51	0.26	1.96
5	0.20	0.17	0.71
6	0.37	0.28	1.32
7	0.17	0.17	1.00
8	0.11	0.24	0.46
24	0.49	0.36	1.36
48	0.81	0.33	2.45*

* $P < 0.050$

Table 20 Deviations of total infected cells of TBF-infected sheep after injection with betamethasone (Hour 0 - Hour X)

Hours post-injection	Degrees of freedom	Mean difference (10^8/l)	e	t
1c	10	4.45	1.19	3.74**
2	10	6.37	1.26	5.06***
3	10	6.23	1.23	5.07***
4	10	8.84	2.55	3.47**
5	10	6.65	1.67	3.98**
6	10	11.59	1.82	6.37***
7	10	11.05	1.64	6.74***
8	10	10.03	1.88	5.34***
24	10	2.35	1.67	1.41
48	7	2.90	3.68	0.79

*** $P < 0.001$; ** $P < 0.010$

Table 21 Deviations of total infected cells of TBF-infected sheep injected with normal saline (Hour 0 - Hour X)

Hours post-injection	Mean difference (10^8/l)	e	$t_{(8)}$
1	-1.06	0.97	1.09
2	-1.10	1.19	0.92
3	-1.09	1.37	0.80
4	-2.62	1.28	2.05
5	-1.62	1.82	0.89
6	-2.66	2.47	1.08
7	-3.40	2.20	1.55
8	-2.18	2.06	1.06
24	-2.79	2.99	0.93
48	-4.44	3.81	1.17

Table 22 Deviations of the percentages of infected granulocytes after injection with betamethasone (Hour 0 - Hour X)

Hours post injection	Degrees of freedom	Mean difference (%)	e	t
1	10	-25.36	4.54	5.59***
2	10	-25.09	4.84	5.18***
3	10	-28.18	4.83	5.83***
4	10	-27.09	5.96	4.54**
5	10	-25.09	4.79	5.24***
6	10	-17.91	5.89	3.04*
7	10	-16.00	4.72	3.39**
8	9	-30.00	16.26	1.85
24	10	- 7.73	7.86	0.98
48	6	-18.57	7.05	2.63*

*** $P < 0.001$; ** $P < 0.010$; * $P < 0.050$

Table 23 Deviations of the percentages of infected granulocytes after injection with normal saline (Hour 0 - Hour X)

Hours post injection	Degrees of freedom	Mean difference (%)	e	t
1	8	-6.56	3.11	2.11
2	8	-0.37	3.06	0.11
3	8	-0.33	3.57	0.09
4	8	-3.78	4.78	0.79
5	8	-1.89	5.82	0.17
6	8	-4.11	6.55	0.63
7	8	7.11	6.00	1.19
8	8	-3.33	5.10	0.65
24	8	-6.56	8.67	0.76
48	7	-12.13	10.24	1.18

Table 24 Comparisons of the titres of antigens prepared before and after culture of infected blood

Hours of culture	Degrees of freedom	Mean difference ($\log_2$)	e	t
0-4	7	1.00	0.46	2.17
0-8	8	1.56	0.41	3.88**
0-24	24	2.20	0.29	7.53***
4-8	7	0.50	0.19	2.63*
4-24	7	1.60	0.56	2.90*
8-24	8	1.00	0.58	1.72

*** $P < 0.001$

** $P < 0.010$

* $P < 0.050$

DISCUSSION

Treatment of sheep infected with tick-borne fever with the corticosteroid, betamethasone, resulted in significant clinical and haematological changes. The most dramatic change was the reduction of rectal temperature. Although the fall was transient, the results indicated that corticosteroids might be useful drugs in the treatment of tick-borne fever.

Betamethasone increased the total leucocytes significantly and this increase of the total leucocytes was essentially due to the increase in the granulocytes. In contrast a transient, but less marked, reduction in lymphocytes was observed following injection.

Corticosteroid-induced neutrophilia is thought to be either due to reduced egress of cells from circulation or due to an influx of new cells from the bone marrow reserve (Mishler, 1977). The results in this experiment indicate that the neutrophilia was essentially due to an influx of new cells from the bone marrow reserve (BMR). This was borne out of the fact that more immature granulocytes were observed following injection of betamethasone. There were clear indications that the cells which came from the BMR were not infected prior to their arrival at the peripheral blood. This was supported by my finding that the dramatic increase in the number of granulocytes was accompanied by a reduction in the percentage of infected cells. The percentage of infected granulocytes continued to rise with time. My findings agree with Snodgrass's (1974) findings that bone marrow was not infective

before the peripheral blood.

Taylor and his colleagues (1941) had observed that bacterial endotoxins induced neutrophilia in sheep infected with TBF and claimed that induction of neutrophilia suggested that TBF did not cause marrow exhaustion. The findings in the present study confirmed that the ability of animals to respond by mobilization of reserve neutrophils was not affected by TBF.

Antigens prepared from leucocytes collected six hours after infected sheep were injected with betamethasone had significantly higher titres than those prepared from leucocytes collected before injection. Moreoever, when infected blood was cultured with HEPES-buffered medium 199 for 24 hours a further increase in the titre of antigens was achieved. Separation of infected granulocytes by density gradient centrifugation increased antigen titres but not significantly. Although the separation of granulocytes eliminated the mononuclear cells and, therefore, increased the concentration of granulocytes it was accompanied by a significant overall loss of infected cells.

The findings in the present study suggested that a combination of the first two methods can resolve the problem of antigen yields. For successful improvement of antigen yields infected animals should be injected with a corticosteroid at the peak period of parasitaemia; leucocytes collected six hours after injection should then be cultured for 24 hours at $37^{\circ}C$.

CHAPTER SEVEN

HUMORAL ANTIBODIES AND IMMUNITY

INTRODUCTION

Little is known of the relationship between humoral antibodies and resistance to reinfection by C. phagocytophila. Various workers have shown that while some animals remained immune to reinfections for long periods others lost their immunity after a few months (Tuomi, 1967[a], Scott, 1978). McEwen (1947) speculated that the resistance of young lambs to infections with TBF might be due to maternal antibodies passed through the colostrum. Snodgrass (1974), on the other hand, thought that the CF antibodies might be nothing more than an indication of past experiences of the disease but his data do not substantiate his arguments. The present work was undertaken to investigate the possible correlation of CF antibodies and resistance to reinfection.

MATERIALS AND METHODS

Experimental Infections

Adult sheep obtained from tick-free areas were inoculated with the OS strain as described in Chapter Two. The sheep were kept under observation for 14 to 21 days and the clinical and haematological parameters estimated and recorded as described previously.

Collection of Sera

Sera were collected from all sheep before infection and every week for eight weeks after infection and every month thereafter. The sera were stored at -20°C until required. Before use they were heat-inactivated at -56°C for 30 minutes and treated with guinea pig

complement to reduce anti-complementary activity as described in Chapter Five.

Complement Fixation Test

The complement fixation test was carried out as described previously in Chapter Five. Enough antigen was pooled and stored at $-20^{\circ}C$ and the same dilution of antigen used throughout the experiment.

Design of Experiments

Preliminary results indicated that sheep which were challenged when their reciprocal CF antibody titres were lower than 2^4 reacted with parasitaemia. Sheep which had experienced a primary infection with C. phagocytophila were, therefore, divided into three groups:

Group One. Fourteen sheep in this group were challenged when their reciprocal CF titres were more than 2^4.

Group Two. Ten sheep in this group were challenged as soon as their reciprocal CF titres had reached 2^4.

Group Three. Eleven sheep in this group were challenged when their reciprocal CF titres were below 2^4.

All the sheep were kept under observation for at least 14 days after challenge. Blood samples were taken before challenge and every day thereafter. The clinical and haematological parameters were estimated as described previously (Chapter Two). Serum samples were taken immediately before challenge and every week thereafter for three weeks. The CF antibody titres were estimated

as described elsewhere (Chapter Five). Some of the prechallenge sera were fractioned into IgM and IgG as described in Chapter Eight and separately tested for CF activity.

Analysis of Data

Means and standard deviations were calculated according to conventional methods. Clinical reactions to primary and secondary infections were compared by analysing the pair differences of each parameter by Student's t-test for significance. Deviations of CF antibody titres after challenge inoculations were similarly analysed. Independent groups were compared by analysing their means and standard deviations by Student's t-test.

RESULTS

Clinical Reactions to Challenge Inoculations

None of the sheep which were challenged when their reciprocal CF titres were above 2^4 (Group One) reacted with fever or parasitaemia (Appendix Table 28). Six of the ten sheep which were challenged when their reciprocal CF titres were 2^4 (Group Two) reacted clinically while three did not (Appendix Table 28). All but one of the eleven sheep which were challenged when their reciprocal CF titres were below 2^4 (Group Three) reacted with parasitaemia (Appendix Table 28). Some sheep reacted with a short period of parasitaemia and fever while others showed only a short period of parasitaemia without fever (Appendix Table 28).

Comparisons of Primary and Secondary Reactions

Incubation period. The incubation periods of secondary reactions were significantly longer than those of primary reactions (Figures 17 and 18, Table 25, Appendix Table 29).

Fever. The duration and magnitude of febrile reactions were significantly lower in secondary reactions than primary reactions (Figure 17, Table 25, Appendix Tables 30 and 31). The peak temperatures of primary reactions were also significantly higher than those of secondary reactions (Table 25, Appendix Table 32).

Parasitaemia. The durations and magnitudes of parasitaemia were significantly lower in the secondary reactions than in the primary reactions (Figure 18, Table 25, Appendix Tables 30 and 31). The numbers of infected cells at peak parasitaemia of primary infections were significantly more than those of secondary reactions (Table 25, Appendix Table 32).

Leucopaenia. No significant haematological changes were observed in those sheep which did not react to challenge inoculations. The sheep which reacted to challenge inoculations had some degree of neutropaenia and lymphocytopaenia. The nadir of the neutropaenia of secondary reactions was higher than the nadir of primary reactions but their difference was not statistically significant (Table 25, Appendix Table 33). The day in which the lowest number of neutrophils were recorded was not significantly different in the secondary reactions (Table 25, Appendix Table 33). The nadir of lymphocytopaenia of primary reactions was significantly lower than that of the secondary reactions (Table 25, Appendix Table 33).

The day on which the lowest numbers of lymphocytes were recorded in the secondary reactions was not different from that of primary reactions (Table 25, Appendix Table 33).

Antibody Responses to Challenge Inoculations

The antibody response of sheep that did not react to challenge inoculations was characterised by a slight increase one week after inoculation. The increases were statistically significant two weeks post-challenge but were not significant three weeks post-challenge (Table 26, Appendix Table 34). In contrast, sheep which reacted clinically after challenge inoculations had significant rises in their CF antibody titres as early as one week after challenge (Table 26, Appendix Table 35). The increases in antibody titres were statistically very significant after one, two and three weeks post-challenge. The increases in CF antibody levels of the sheep which reacted were significantly higher than the corresponding increases in the sheep which did not react after one, two and three weeks post-challenge ($t_{30} = 4.70$, $p < 0.001$; $t_{30} = 7.56$, $p < 0.001$; and $t_{27} = 5.63$, $p < 0.001$ respectively).

Comparisons of Antibody Responses to Primary and Challenge Infections

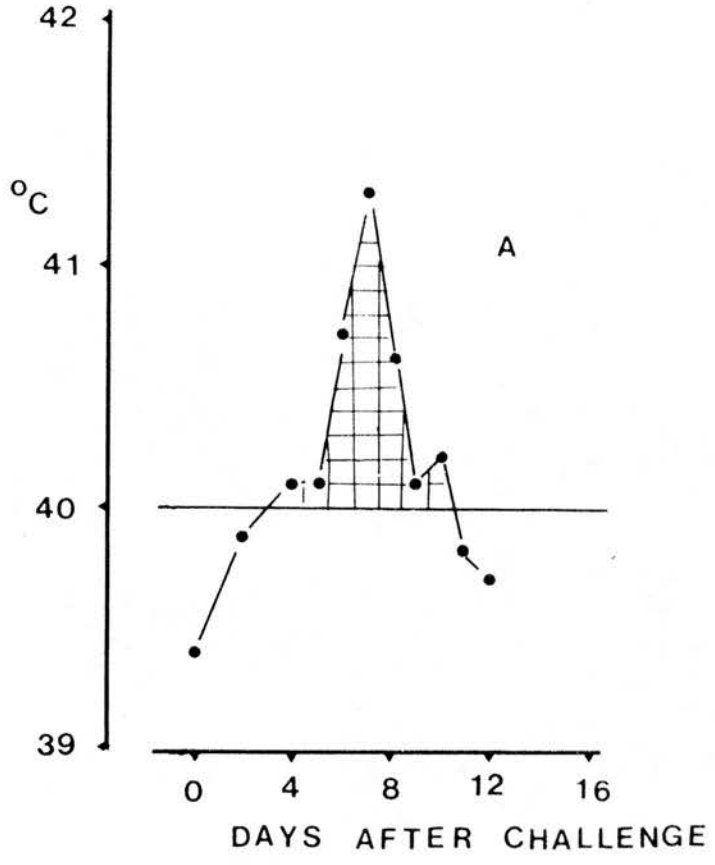
The antibody responses to challenge infections were faster than the responses to primary infections. In primary infections the CF antibodies were usually detected two weeks after inoculation but in secondary infections it was found that the CF titres were significantly higher than pre-challenge titres one week after challenge. The antibody titres of secondary reactions were lower than primary infections. They were not significantly lower than

those that follow primary reactions after two weeks but three weeks after challenge the CF titres were significantly lower than those that follow primary infections ($t_{37} = 1.33$, $p > 0.20$ and $t_{37} = 2.69$, $p < 0.05$ respectively; Table 11 and Appendix Table 35).

Figure 17 Comparison of the febrile reactions of primary and secondary infections

A - secondary reaction

B - primary reaction


42
°C
41
40
39
A
0
4
8
12
16
DAYS AFTER CHALLENGE

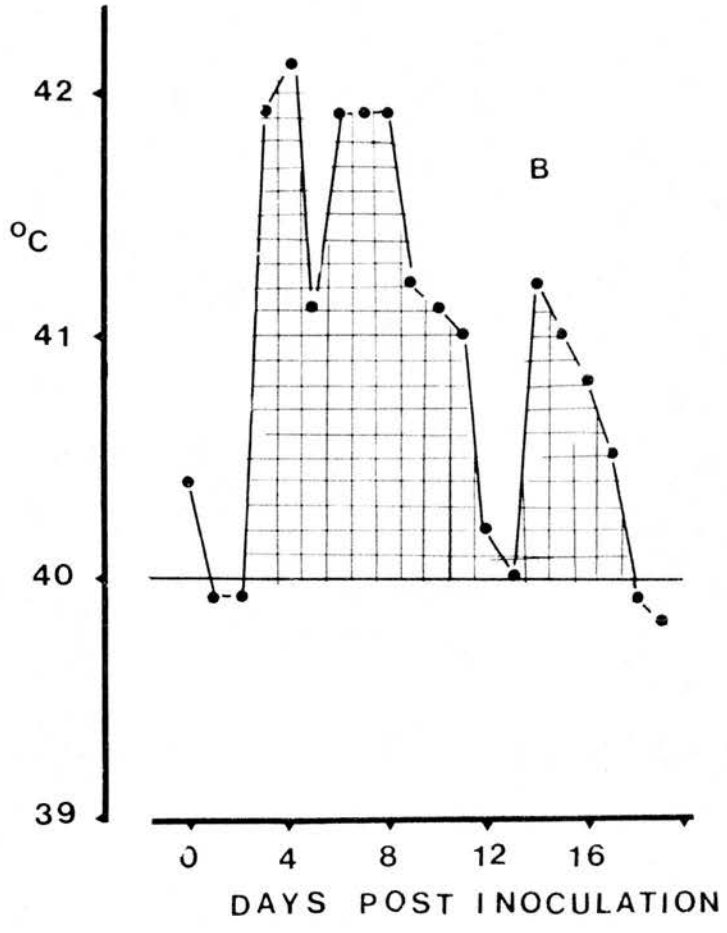
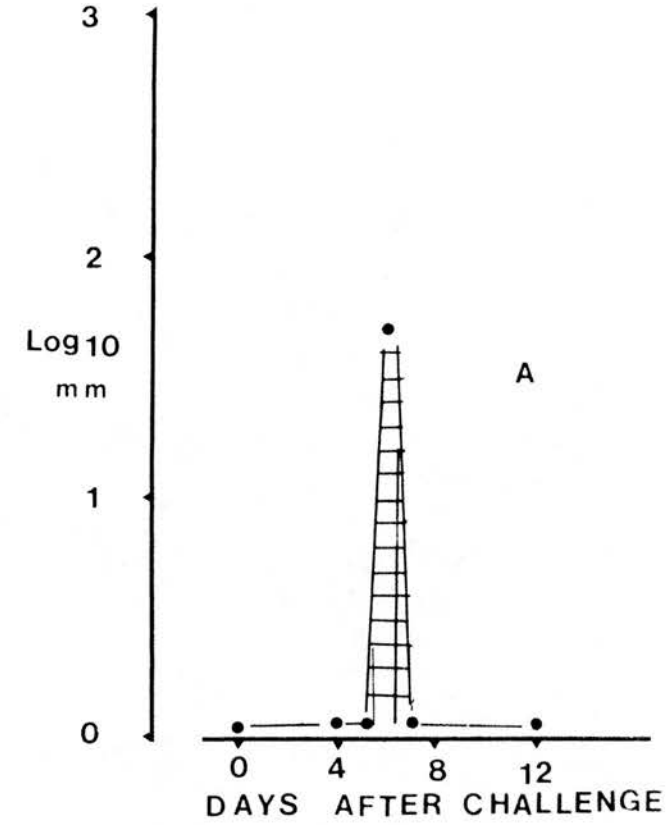

42
°C
41
40
39
B
0
4
8
12
16
DAYS POST INOCULATION

Figure 18 Comparison of the prepatent period and the magnitudes and durations of parasitaemias of primary and secondary infections

A - secondary reaction

B - primary reaction


3
2
Log10
mm
1
0
A
0
4
8
12
DAYS AFTER CHALLENGE

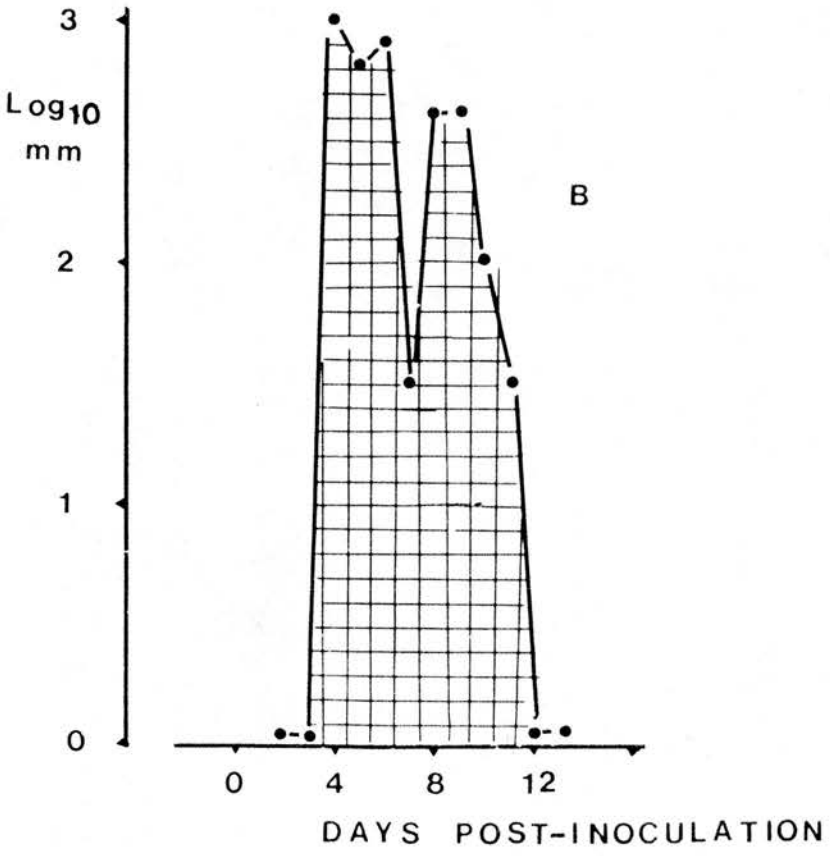

3
Log10
mm
2
1
0
B
0
4
8
12
DAYS POST-INOCULATION

Table 25 Comparison of the clinical parameters of primary and secondary reactions

Parameter	Degrees of freedom	Mean difference	e	t
Incubation period (days)	8	2.2	0.40	5.50***
Peak fever (^{o}C)	8	0.99	0.23	4.30**
Duration of fever (days)	8	5.30	0.97	5.46**
Magnitude of fever (mm^2)	8	1869.00	330.00	5.66***
Peak parasitaemia ($10^3/mm^2$)	8	1.51	0.29	5.21**
Duration of parasitaemia (days)	8	3.56	0.75	4.75**
Magnitude of parasitaemia (mm^2)	8	2722.00	375.00	7.26***
Nadir of neutropaenia ($10^3/mm^2$)	7	0.23	0.10	2.30
Day of nadir of neutropaenia	7	0.88	0.99	0.89
Nadir of lymphocytopaenia ($10^3/mm^2$)	7	0.96	0.24	4.00**
Day of nadir of lymphocytopaenia	7	1.38	0.75	1.84

*** $P < 0.001$; ** $P < 0.010$

Table 26 Significance of the differences of CF titres of sera obtained before and after challenge inoculations

	Weeks post-challenge	Degrees of freedom	Mean difference (log_2)	e	t
Reactors	1	13	2.36	0.34	6.94***
	2	13	3.43	0.27	12.70***
	3	13	2.86	0.40	7.15***
Non reactors	1	17	0.39	0.26	1.50
	2	17	0.56	0.26	2.15*
	3	14	0.20	0.26	0.77

*** $P < 0.001$; * $P < 0.050$

DISCUSSION

Sheep experimentally infected with C. phagocytophila developed detectable CF antibodies within two weeks after infection. Similar findings have been reported by Snodgrass and Ramachandran (1971). The CF antibodies reached peak levels between two and three weeks. They continued to be detected at lower levels for a considerable period.

Sheep which were challenged when their reciprocal CF antibody titres were higher than 2^4 did not react clinically irrespective of the length of time between primary infection and challenge inoculation. In contrast, animals which were challenged as soon as their reciprocal antibody levels had dropped to below 2^4 reacted either with a mild parasitaemia and fever or with a mild parasitaemia only. These findings suggest that there is some relationship between CF antibodies and resistance to reinfection. The increases in the CF antibody titres of animals which did not react to challenge inoculations were very significantly smaller than the increases of the titres of those animals which reacted (Table 26). This may be associated with the carrier state. C. phagocytophila and other animals rickettsias such as E. canis and Cowdria ruminantium are characterised by the persistence of the organisms for long periods after the cessation of clinical reactions. Immunity to these organisms have been attributed to a state of premunition followed by a gradually declining sterile immunity (Neitz, Alexander and Adelaar, 1947).

If the sheep which did not react to challenge inoculations were in a state of premunition, it is conceivable that the introduced

challenge antigen did not add significantly to the antigenic mass already present in the carrier animal which would explain the lack of big increases in antibody titres. The present finding also suggested that some animals had sterile immunity. The mildness of the clinical reactions and the speed of antibody response support this hypothesis. The longer incubation periods of secondary reactions suggested that the low levels of antibodies neutralized some organisms and by so doing delayed clinical disease. The brevity of clinical reactions and parasitaemia may be due to the rapid anamnestic antibody production by committed memory cells after being stimulated by challenge inoculations. The last hypothesis is supported by the finding that the antibody titres were significantly increased as early as one week after challenge and that the mean febrile period and mean parasitaemic period were significantly shorter in the secondary reactions than the primary reactions.

The role of humoral antibodies in the immunity against animal rickettsias is not very clear. While some workers have contended that humoral antibodies have no protective role (Du Plessis, 1970), others have presented evidences which suggested that humoral antibodies play an important role in protective immunity. Ristic (1978) speculated that immunity to animal rickettsias might be dependent on the synergism of humoral and cell-mediated immune responses. Both humoral and cell-mediated immune responses have been demonstrated in infections produced by E. canis (Nyindo, Huxsoll, Ristic, Kakoma, Brown, Carson and Stephenson, 1980) and anaplasmosis (Murphy, Osebold and Aalund, 1966; Carson, Sells and Ristic, 1977),

but whether a synergistic effect of cell-mediated and humoral immune responses occur has not been established.

The killing of parasites that enter macrophages is believed to be the result of fusion by lysosomes with phagosomes containing the organism (Hirsch, 1972). C.phagocytophila and E. equi parasitize the granular leucocytes and E. canis invades mononuclear cells. These organisms reside in cytoplasmic vacuoles. How they evade digestion by the phagocytes is not known. Although Tuomi and von Bonsdorff (1966) failed to demonstrate any granular material which could indicate lysosomal activity, I found, not infrequently, degenerated particles inside vacuoles and Kraus and his colleagues (1972) had reported that they observed leucocyte granules discharged from the cytoplasm into the vacuoles containing Cytoecetes ondiri. It is conceivable that these organisms possess some mechanism by which the activity of the lysosomes is inhibited or greatly reduced at least during the early phases of infection. Such inhibitory activity has been demonstrated in other organisms such as the Chlamydia (Friis, 1972) and Toxoplasma gondii (Jones, 1974).

Recent work on other rickettsias indicated that antibodies might affect the organisms in such a way that they no longer were capable of withstanding lysosomal action and digestion. Gambril and Wisseman (1973), for instance, found that Rickettsia mooseri preincubated with immune serum was rapidly phagocytosed and destroyed by monocyte-derived human macrophages which, by themselves, would not destroy the organism. Lewis, Hill and Ristic (1978) found that treatment of E. canis with immune canine serum

suppressed the organism's rate of infection of normal canine macrophages in vitro. It is conceivable that in the early phases of infection the monocytes and granulocytes act favourably for the organism. They actively phagocytose it but due to the organism's ability to reduce lysosomal activity and destruction, allow it to multiply unhindered in the protective shield created by the cytoplasmic vacuole. As antibodies are produced in abundance they coat cell-free organisms which are then rapidly phagocytosed and digested probably by a mechanism similar to that demonstrated in other facultative intracellular organisms (Friis, 1972; Gambril and Wisseman, 1973; Jones, 1974).

CHAPTER EIGHT

IMMUNOGLOBULINS OF SHEEP INFECTED WITH *CYTOECETES PHAGOCYTOPHILA*

INTRODUCTION

Infections with C. phagocytophila are followed by the production of complement fixing antibodies (Snodgrass and Ramachandran, 1974; Chapters Five and Seven). The types of immunoglobulins involved however, are not known. Scott and Koske (1976) reported that there was no significant change in the total serum protein of sheep infected with C. phagocytophila but their studies were limited to the period of clinical reaction. Burghen, Beisel, Walker, Nims, Huxsoll and Hilderbrandt (1971) reported that canine ehrlichiosis was followed by marked hypergammaglobulinaemia and Buhles, Huxsoll and Ristic (1974) suggested that it might be due to immunopathologic reactions. They thought that hypergammaglobulinaemia might be caused by the persistence of the organism resulting in an excessive production of antibodies. Although the pancytopaenia that accompanies canine ehrlichiosis is not a feature of TBF, the two diseases are caused by similar organisms which persist in the host for long periods. Furthermore, TBF has been accused of depressing the resistance of infected animals to other diseases and conditions but the mechanism of the alleged immunosuppression has not been clarified.

The present study was undertaken to investigate the possible changes of serum proteins of sheep infected with TBF over a period of two months and to study the CF activity of individual classes of immunoglobulins over long periods after infection.

MATERIALS AND METHODS

Collection of Serum Samples

Serum samples were collected from 12 sheep before infection with the OS strain of C. phagocytophila and every week thereafter for eight weeks. Eight control sheep were injected with one ml of normal saline and were similarly bled for serum for eight weeks. Serum samples were stored at -20°C in aliquots of four ml until required.

Estimation of Serum Protein Levels

Total protein levels were estimated by the biuret technique (Lowry, et al., 1951). Sequential changes in the components of the serum protein were determined by electrophoresis. Densitometric quantitations were carried out on stained slides with a phoroscope (Millipore Corporation Ltd.) and individual concentrations calculated from the total proteins.

Fractionation of Immunoglobulins

The immunoglobulins were fractionated by chemical precipitation and gel-filtration and ion-exchange chromatography.

Precipiration by Dialysis Against Tap-water

The serum samples were loaded into visking tubes and dialysed against tap-water, adjusted to pH 6.1 at 4°C, for 72 hours. This resulted in the precipitation of the euglobulins which included most of the IgM. The precipitated material was centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was separated and used as a starting material for isolating the IgG while the precipitate

was dissolved in small quantities of 0.1M Tris-HCl buffer at pH 8.0 and used for further purification of IgM.

Preparation of Chromatographic Columns

Both gel-filtration and ion-exchange chromatography were used to purify the IgM and IgG fractions.

Gel-filtration chromatography. This was carried out in columns packed with Sephadex-G200 (Pharmacia Ltd., London). Before packing them into a 100 cm by 2.5 cm column approximately 30 g of Sephadex-G200 was allowed to swell at $4^{\circ}C$ for three days in two litres of 0.1M Tris-HCl buffer + 1M NaCl at pH 8.0 and 0.02 percent sodium azide (Jonas, 1969; Shubber, 1978). Excess buffer was removed, leaving the slurry which was subsequently degassed to avoid the formation of bubbles. The gel-bed was then allowed to pack under a flow rate of 20 ml per hour for three days with 0.1M Tris - HCl buffer + 1M NaCl at pH 8.0. The top-piece was then fitted and an upward flow operated using a peristaltic pump to give a flow rate of 20 ml per hour for another three-day period,

The homogeneity of the gel-bed was checked by running 2 mg per ml blue dextran 2000 (Pharmacia Fine Chemicals). The gel was washed by topping the reservoir with buffer.

Ion-exchange chromatography. The pre-swollen micro-granular ion-exchanger, diethyl aminoethyl (DEAE) cellulose (Whatman-DE-52) was packed into a 40 cm by 2.5 cm column. The packing process was as follows: the required amount of the DE-52 was placed in the acid component (KH_2PO_4, 0.5 M) of the phosphate buffer, using higher concentrations if the pH did not fall below 4.5. The slurry

was then put into stoppered Buchner flask and degassed under pressure with a continuous magnetic stirring. Then the basic component (Na_2HPO_4, 0.5 M) was added to the slurry to make the final pH 7.6. The slurry was filtered through a Whatman 54 filter paper under a reduced pressure provided by a venturi pump. The slurry was washed with 0.1M phosphate buffer at pH 7.6 twice and filtered several times using 0.1M phosphate buffer at pH 7.6 until the pH of the filtrate was the same as that of the equilibrium buffer (Shubber, 1978).

The gel was finally allowed to settle in the starting buffer and before pouring it into the column gently stirred. The column was left in draught-free area and the effluent allowed to run out until a few ml of buffer were left on the top. After 24 hours the top-piece was attached and the column moved into the LKB unit at 10°C to 12°C.

Isolation of IgM

The precipitate formed following a 72-hour dialysis against tap-water served as the starting material for the purification of the IgM fraction. The precipitate was dissolved in 0.1M Tris-HCl buffer at pH 8.0. Two ml of the dissolved euglobulins were applied to the Sephadex G-200 column and allowed to fractionate at a flow rate of 20 ml per hour with Tris-HCl buffer + 1M NaCl at pH 8.0 as an eluent. All the protein containing peaks as detected at 280 nm wavelength by an LKB unit and collected by a fraction collector were pooled. They were concentrated by dialysis against 40 percent polyethylene glycol (Carbowax, British Drug House) and

reconstituted to the original volume of the serum sample with veronal buffer and stored at -20°C until required.

Isolation of IgG

The supernatant left after the precipitation of the euglobulins was used as a source of IgG. The supernatant was precipitated with a 50 percent saturated ammonium sulphate adjusted to pH 8.2 with ammonia (specific gravity 0.8). Samples were agitated with a magnetic stirrer and equal amounts of saturated ammonium sulphate added in drops. The precipitate was left overnight at 4°C. Then the material was centrifuged at 2000 *g* for 30 minutes at 4°C and the supernatant discarded. The precipiate was dissolved with distilled water to make the original volume of the sample and the process of precipitation repeated twice. The final precipitate was dissolved in distilled water, loaded into visking tubes and dialysed against tap-water first and against phosphate buffer at pH 7.6 for another 24 hours. This provided the starting material for the purification of the IgG by ion-exchange chromatography. The IgG fraction was loaded onto the column and eluted with 0.01M, 0.02M and 0.05M phosphate buffer at pH 7.6 (Reid, Docherty and Dawson, 1971) and collected with a fraction collector as described earlier. All the peaks which contained proteins were pooled. No attempt was made to separate IgG1 and IgG2. The pooled samples were concentrated by dialysis against polyethylene glycol and reconstituted to original volume of the serum sample with veronal buffer and stored at -20°C until required.

Immunoelectrophoresis

The purity of the fractionated immunoglobulins was tested by immunoelectrophoresis. After preparing wells and troughs in slides previously coated with 1.5 percent special agar (Oxoid Ltd.) the isolated fractions were put into appropriate wells and the slides placed in a tray of an electrophoretic chamber which was filled with barbital buffer, pH 8.6. Moist wicks made of filter paper were attached to the edges of the gel and connecting it with the buffer of the electrophoretic chamber. A current of 100 volts was then applied for 60 minutes. The plates were removed and placed in moist chambers and anti-sheep whole serum prepared in a rabbit added to the trough and diffusion allowed to proceed at room temperature or at $4^{o}C$ overnight. The plates were washed with saline and the precipitation lines observed for their typical arcs which characterise IgG and IgM.

Treatment of Sera with 2-Mercaptoethanol

Sera were treated with 2-mercaptoethanol (2-ME) by adding 0.1 ml of serum to 0.1 ml of a 2-ME dilution that would give a final concentration of 0.2M of 2-ME (Reid et al., 1971). Control samples were mixed with veronal buffer. The mixture was left at room temperature for two hours and dialysed against large volumes of veronal buffer for 24 hours. Each sample was then tested for CF activity.

Complement Fixation Test

The sera were tested for CF activity before and after fractionation and before and after treatment with 2-ME according to the

methods described previously in Chapter Five.

Analysis of Data

Changes in the patterns of total proteins and individual components were analysed by comparing the pair-difference of pre- and post-inoculation values by the Student's t-test. Comparisons of CF activity of the IgM and IgG fraction were also based on the analysis of pair-differences. The differences of the CF titres of sera before and after treatment with 2-ME were similarly analysed.

RESULTS

Serum Protein Levels

Serum protein levels of 12 sheep infected with TBF were studied for eight weeks. At the time of inoculation the mean serum protein level was 65.2 ± 8.3 g per litre. The protein levels after inoculation with C. phagocytophila varied from 59.7 ± 14.3 g per litre one week after inoculation to 80.8 ± 18.7 g/l five weeks after infection. The reduction observed one week after infection was not statistically significant but the increases observed four, five and six weeks after infection were significant (Table 27, Appendix Table 36). The protein levels of eight control sheep which were not infected with TBF showed no significant changes during the same period of study (Table 28, Appendix Table 37).

Electrophoresis of serum samples resulted in a clear separation into five easily discernible bands: albumin and alpha 1, alpha 2, beta and gamma globulins. The total globulins of infected sheep were increased two weeks after infection. The increases were

statistically significant after two, four, five, six and eight weeks post-inoculation (Figure 19, Table 29, Appendix Table 38). In contrast there was no significant change in the total globulins of control sheep (Table 30, Appendix Table 39). There were no statistically significant changes in the albmin, and the alpha 1 alpha 2 and gamma globulins of infected or control sheep (Tables 31 to 38, Appendix Tables 40 - 47) but the beta globulin levels of infected sheep were marginally increased and these increases were statistically significant on the sixth and eighth weeks post-inoculation (Figure 20, Table 39, Appendix Table 48). In contrast, there were no significant changes in the control sheep's beta globulins (Table 40, Appendix Table 49).

Immunoglobulin Classes and CF activity

Dialysis of serum samples against tap-water resulted in the precipitation of the euglobulins and this included the IgM. The IgM separated well with Sephadex G-200, separation of two ml quantities being completed in about ten hours. There were usually two peaks but some samples had only one peak made up of ascending and descending concentrations.

Because the serum samples were too small, no attempts were made to separate the IgG into IgG1 and IgG2. Almost all of the IgG separated with 0.05M phosphate buffer but some samples had very small peaks with 0.02M phosphate buffer.

Both IgM and IgG fractions of immune sera showed strong CF activity against antigens of C. phagocytophila. The IgM fraction was dominant for the first few weeks after infection (Figure 21,

Table 41). However, the IgG fraction also showed CF activity as early as two weeks post-inoculation but the CF titres in the IgG fractions were lower than those of the IgM fractions. The highest CF titres of the IgM fractions were observed 2.70 ± 0.95 weeks post-inoculation while the highest CF titres of the IgG fractions were observed 4.60 ± 2.01 weeks post-inoculation (Appendix Table 50). The CF titre of the IgG fractions became higher than the CF titre of the IgM fractions about the sixth week post-inoculation (Figure 21). Although the CF titres of the IgM fractions declined with time, CF activities were still detected for more than 12 months after infection. Pre-challenge sera of some sheep were fractionated into IgM and IgG and their CF activities studied. The mean reciprocal CF titre of the IgM and IgG fractions of nine sheep which did not react to challenge inoculations were $2^{4.33 \pm 1.00}$ and $2^{6.00 - 1.12}$ respectively before inoculation and the patterns did not change significantly after challenge (Table 42). In contrast, the CF activity of pre-challenge sera of ten sheep which reacted to challenge inoculations were lower, the reciprocal titre for the IgM fraction being $2^{1.6 \pm 1.17}$ and the reciprocal titre of the IgG fraction being $2^{3.1 \pm 1.19}$. The CF titres of those sheep which did not react to challenge inoculations were in general higher than those which reacted but the IgM fractions had very high CF activity in those which did not react compared to the minimal levels of CF activities of the IgM fraction of those which reacted.

Effects of 2-ME on CF Titre of Immune Sera

The CF activity of sera was significantly reduced after

treatment with 2-ME for at least seven weeks after infection (Table 43, Appendix Table 51). There were reductions even after eight weeks but the reductions were not statistically significant ($t_7 = 1.32$, $p > 0.20$). When the sera of three sheep which showed CF activity one week after infection were treated with 2-ME, there was no CF activity (Appendix Table 51).

Figure 19 Mean total globulins and CF antibody titres of sera from sheep infected with *C. phagocytophila*

A - CF titre

B - total globulin levels

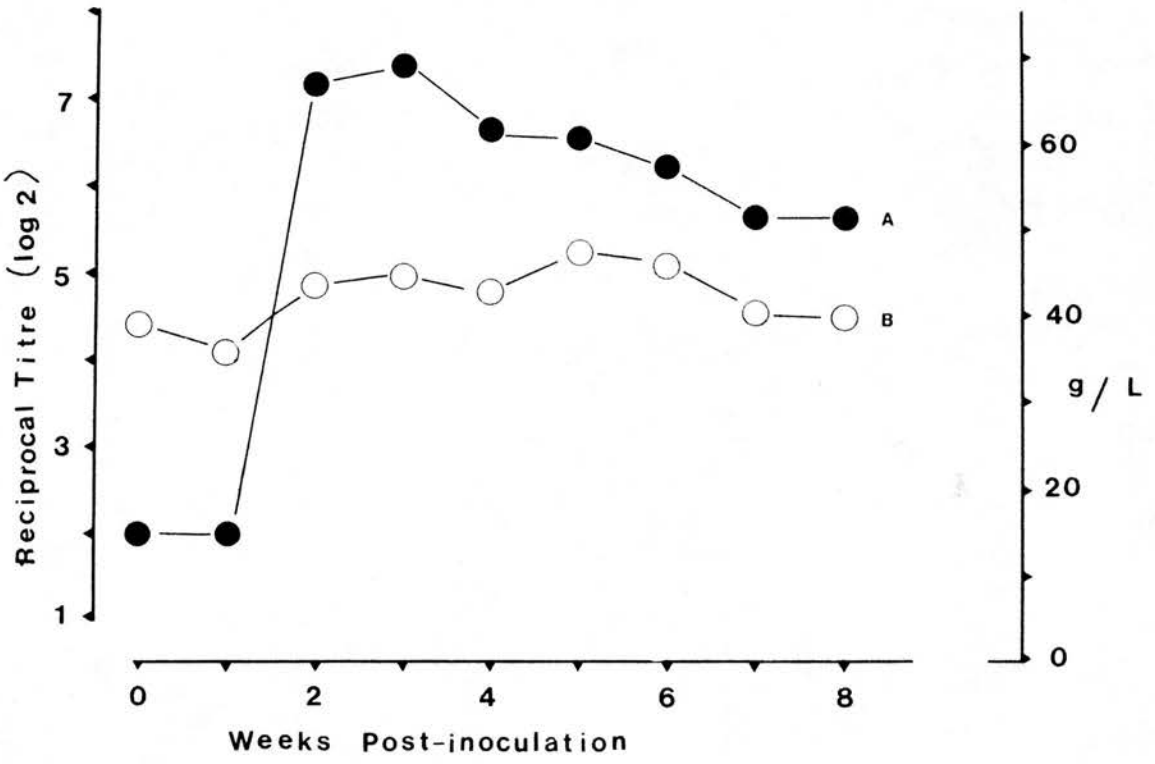

Reciprocal Titre (log 2)
7
5
3
1
0
2
4
6
8
Weeks Post-inoculation
A
B
60
40
g / L
20
0

Figure 20 Mean beta globulin levels and CF antibody titres of sera from sheep infected with *C. phagocytophila*

A - CF titre

B - beta globulin levels

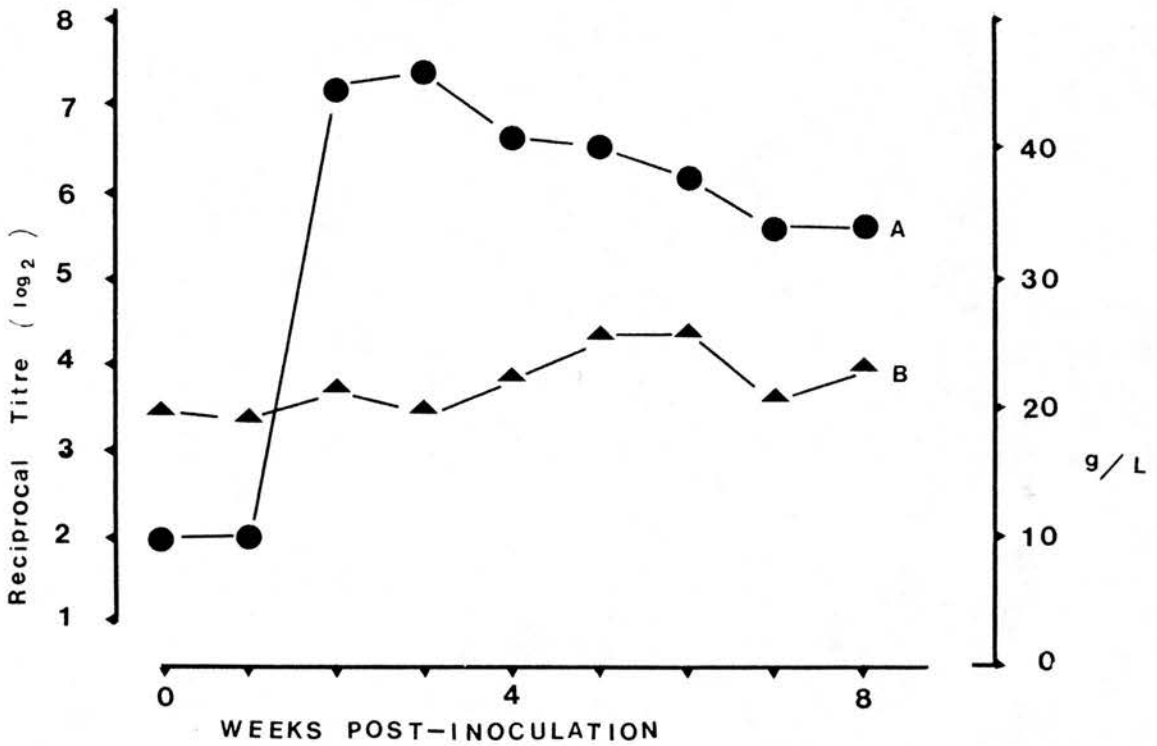

8
7
6
5
4
3
2
1
Reciprocal Titre (log2)
40
30
20
10
0
g/L
A
B
0
4
8
WEEKS POST-INOCULATION

Figure 21 Mean CF antibody titres of the IgM and IgG fractions of sequential sera from infected sheep

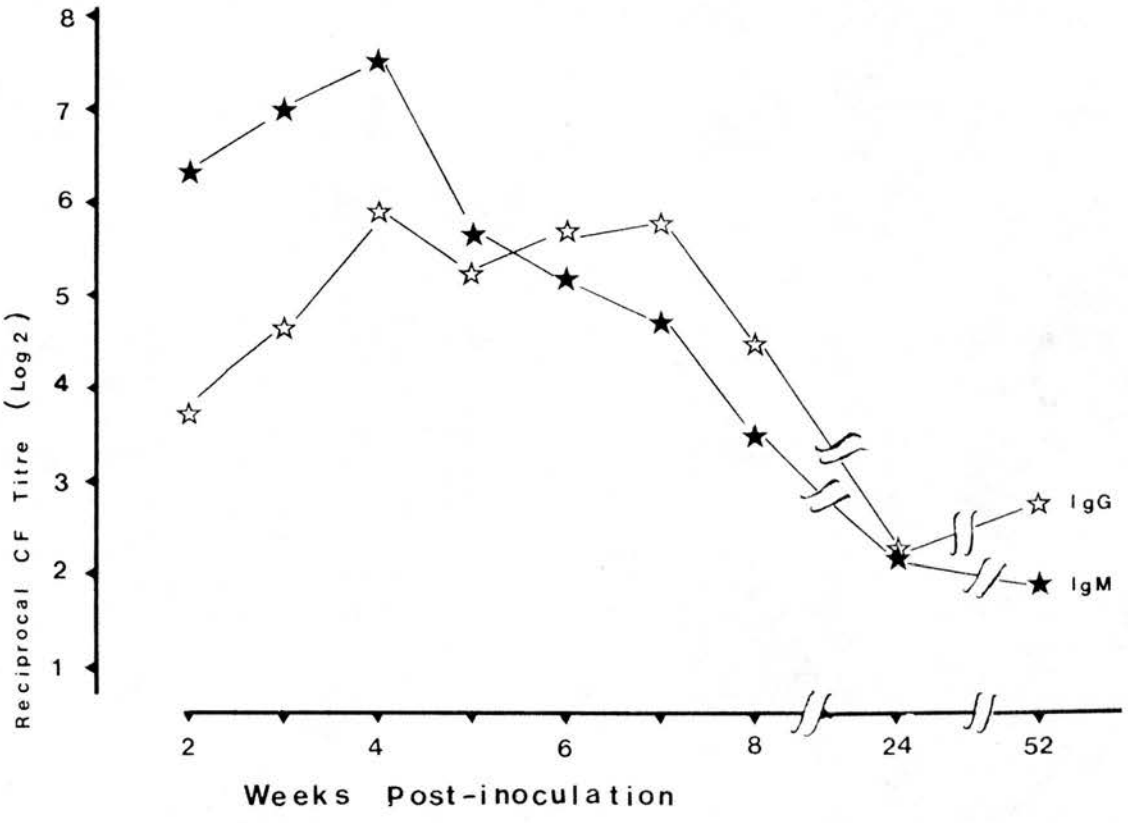

8
7
6
5
4
3
2
1
Reciprocal CF Titre (Log 2)
IgG
IgM
2
4
6
8
24
52
Weeks Post-inoculation

Table 27 Deviations of serum proteins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Mean difference (g/l)	e	$t_{(11)}$
1	-0.56	0.36	1.56
2	0.28	0.43	0.65
3	0.75	0.42	1.79
4	0.97	0.33	2.94*
5	1.57	0.63	2.49*
6	1.46	0.45	3.24**
7	0.36	0.20	1.80
8	0.55	0.35	1.57

** $P < 0.010$; * $P < 0.050$

Table 28 Deviations of serum proteins of uninfected sheep after injection with normal saline (Day 0 - Week X)

Weeks post-infection	Mean difference (g/l)	e	$t_{(7)}$
1	0.50	0.24	2.08
2	-0.20	0.22	0.91
3	0.15	0.39	0.38
4	0.18	0.31	0.58
5	0.25	0.51	0.49
6	0.34	0.41	0.83
7	0.64	0.58	1.10
8	0.59	0.52	1.13

Table 29 Deviations of total globulins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	-0.18	0.17	1.06
2	10	0.65	0.22	2.95*
3	11	0.56	0.28	2.00
4	10	0.51	0.22	2.32*
5	11	1.00	0.33	3.03*
6	11	0.73	0.27	2.70*
7	10	0.35	0.28	1.25
8	9	0.29	0.12	2.42*

* $P < 0.050$

Table 30 Deviations of total globulins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.28	0.26	1.08
2	7	0.28	0.26	1.08
3	7	0.14	0.26	0.54
4	7	0.07	0.46	0.15
5	7	0.13	0.43	0.30
6	7	0.16	0.35	0.46
7	6	0.55	0.41	1.34
8	6	0.22	0.36	0.61

Table 31 Deviations of serum albumins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	-0.32	0.21	1.52
2	10	-0.10	0.24	0.42
3	11	0.25	0.14	1.79
4	11	0.49	0.21	2.33*
5	11	0.63	0.33	1.91
6	11	0.48	0.27	1.78
7	10	0.19	0.25	0.76
8	10	0.37	0.37	1.00

* $P < 0.050$

Table 32 Deviations of the serum albumins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.21	0.15	1.40
2	7	0.11	0.14	0.79
3	7	0.28	0.23	1.22
4	7	0.23	0.22	1.05
5	7	0.12	0.15	0.80
6	7	0.18	0.15	1.2
7	7	0.12	0.15	0.80
8	7	0.29	0.26	1.12

Table 33 Deviations of serum alpha 1 globulins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	-0.08	0.10	0.80
2	10	0.21	0.18	1.17
3	11	-0.07	0.13	0.54
4	10	-0.01	0.13	0.08
5	11	0.05	0.16	0.31
6	11	-0.08	0.18	0.44
7	9	-0.02	0.18	0.11
8	9	0.16	0.10	1.60

Table 34 Deviations of serum alpha 1 globulins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.05	0.04	1.25
2	7	0.04	0.02	2.00
3	7	0.03	0.04	0.75
4	7	0.04	0.04	1.00
5	7	0.04	0.06	0.67
6	7	0.06	0.04	1.50
7	6	0.06	0.04	1.50
8	7	0.07	0.05	1.40

Table 35 Deviations of serum alpha 2 globulins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	0.17	0.12	1.42
2	10	0.22	0.15	1.47
3	11	0.20	0.15	1.33
4	10	0.18	0.13	1.38
5	11	0.25	0.14	1.79
6	11	0.19	0.20	0.95
7	9	0.11	0.11	1.00
8	9	0.06	0.11	0.55

Table 36 Deviations of serum alpha 2 globulins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.01	0.03	0.33
2	7	0.01	0.04	0.25
3	7	0.05	0.04	1.25
4	7	0.03	0.08	0.38
5	7	0.05	0.07	0.71
6	7	0.02	0.06	0.33
7	6	0.09	0.04	2.25*
8	7	0.07	0.06	1.17

* $P < 0.050$

Table 37 Deviations of serum gammaglobulins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	0.01	0.05	0.20
2	10	0.07	0.05	1.40
3	11	0.06	0.04	1.50
4	10	0.04	0.04	1.00
5	11	0.06	0.06	1.00
6	11	0.12	0.06	2.00
7	9	0.10	0.07	1.43
8	9	0.11	0.08	1.38

Table 38 Deviations of serum gammaglobulins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.07	0.04	1.75
2	7	0.05	0.07	0.71
3	7	0.03	0.07	0.43
4	7	0.01	0.06	0.17
5	7	0.15	0.07	2.14
6	7	0.05	0.06	0.83
7	6	0.11	0.08	1.38
8	7	0.11	0.10	1.10

Table 39 Deviations of serum beta globulins of TBF-infected sheep from pre-inoculation levels (Day 0 - Week X)

Weeks post-inoculation	Degrees of freedom	Mean difference (g/l)	e	t
1	11	-0.21	0.17	1.24
2	10	0.27	0.19	1.42
3	11	0.04	0.18	0.22
4	11	0.33	0.20	1.65
5	11	0.57	0.29	1.97
6	11	0.61	0.17	3.59**
7	9	0.10	0.18	0.56
8	9	0.36	0.12	3.00*

** $P < 0.010$; * $P < 0.050$

Table 40 Deviations of serum beta globulins of uninfected sheep injected with normal saline (Day 0 - Week X)

Weeks post-injection	Degrees of freedom	Mean difference (g/l)	e	t
1	7	0.16	0.12	1.33
2	7	0.21	0.17	1.24
3	7	0.05	0.12	0.42
4	7	0.14	0.25	0.56
5	7	0.19	0.24	0.79
6	7	0.14	0.22	0.64
7	6	0.30	0.29	1.03
8	7	0.30	0.28	1.07

Table 41 Number of observations (n), means and standard deviation (sd) of reciprocal antibody titres (log_2) of the IgM and IgG fractions of sequential sera

Weeks post-inoculation	IgM n	IgM mean	IgM sd	IgG n	IgG mean	IgG sd
0	10	N		10	N	
1	10	N		10	N	
2	10	6.3	2.58	10	4.2	1.99
3	9	7.0	2.24	9	4.9	2.8
4	9	7.5	2.15	10	6.0	1.63
5	9	5.6	1.59	8	5.3	2.19
6	9	5.3	2.00	8	5.6	1.69
7	9	4.8	1.64	9	5.7	2.4
8	10	3.4	1.78	10	4.5	1.84
26	8	2.3	1.04	8	1.9	1.25
52	7	1.4	0.53	7	2.7	0.95

N = negative sample

Table 42 Mean reciprocal CF titre (log_2) of IgM and IgG fractions of pre- and post-challenge sera from sheep which did not react clinically

Weeks post-challenge	IgM n	IgM mean	IgM standard deviation	IgG n	IgG mean	IgG standard deviation
pre-challenge	9	4.33	1.00	9	6.00	1.12
1	9	4.33	0.50	9	6.67	1.00
2	9	4.00	0.80	9	5.80	1.48

Table 43 Significance of the differences of CF titres of sera before and after treatment with 2-ME

Weeks post-inoculation	Degrees of freedom	Mean difference ($\log_2$)	e	t
1	2	3.0	1.0	3.00
2	8	2.44	0.73	3.35*
3	7	2.38	0.53	4.49**
4	8	1.11	0.48	2.31*
5	8	1.44	0.44	3.27*
6	7	1.00	0.27	3.70**
7	6	1.43	0.48	2.98*
8	7	0.50	0.38	1.32

** $P < 0.010$; * $P < 0.050$

DISCUSSION

During the stages in which antibody is formed at rapid rates, the increases in serum antibody concentrations reflect primarily antibody synthesis (Uhr and Finkelstein, 1967). The initial antibody response to bacterial and viral infections is believed to be characterised by the production of antibodies which are 2-mercaptoethanol sensitive, IgM. This is followed by persistent production of IgG. However, in certain instances production of IgM may persist for long periods. This depends on the type of antigen involved and its duration in the host. Nossal, Ada and Austin (1964), for example, found that particulate antigens favoured the synthesis of IgM. The persistence of the stimulating antigens may also favour the sustained production of IgM (Murphy et al., 1966). Immunosuppressive agents as X-irradiation (Svehag and Mandel, 1964) or 6-mercaptopurine (Sahiar and Schwartz, 1965) have been reported to prolong the period of IgM synthesis.

In the present study CF antibodies against C. phagocytophila were demonstrated in the IgM and IgG classes. The IgM, not unexpectedly, was dominant in the early weeks but what was significant was its persistence. This was probably due to the carrier state. This hypothesis is reinforced by my finding that the increases of the antibody titres of sheep which did not react to challenge inoculations were significantly smaller than the increases of the antibody titres of sheep which reacted (Chapter Seven). An analysis of the CF activity of the pre-challenge sera of the groups of sheep which did not react to challenge inoculation revealed that the IgM class had high CF activity compared to the weak CF activity

of the IgM class of those sheep which reacted. It appears that low grade stimulation of antigen results in the low grade synthesis of IgM for long periods. Weisigar, Ristic and Huxsoll (1975) found that dogs which were carriers of Ehrlichia canis had high titres of antibody in the IgM class for prolonged periods.

Some workers have theorized that the reported hypergammaglobulinaemia of chronic canine ehrlichiosis might be due to the continued antigenic stimulation by the persistence of the organism (Burghen et al., 1971; Buhles et al., 1974) but they did not rule out the possibility that the plasmocytosis and hypergammaglobulinaemia might be due to an auto-immune reaction. However, despite the close relationship between C. phagocytophila and E. canis the pathogenesis of TBF and tropical canine pancytopaenia appear to be very different. The latter is a chronic and fatal disease characterised by a haemorrhagic diathesis (Hilderbrandt, Huxsoll, Walker, Nims, Taylor and Andrews, 1973). In contrast, TBF is an acute disease which rarely terminates fatally and is not characterised by prolonged pancytopaenia or haemorrhagic diathesis. In the present study no evidence of hypergammaglobulinaemia was obtained but there was slight increases in the total globulins and the beta globulins during the period of rapid synthesis of antibodies. Moreover, the hypergammaglobulinaemia of chronic ehrlichiosis has been attributed to an auto-immune reaction (Burghen et al., 1971). Buhles and his colleagues (1974), for example, found that when they treated dogs suffering from chronic ehrlichiosis, the hypergammaglobulinaemia was eliminated but the fluorescent antibody titres were not reduced suggesting that the hypergammaglobulinaemia was

not due to specific antibody synthesis.

In summary, infections of sheep with C. phagocytophila induced a primary antibody response characterised by an initial production of IgM followed by IgG but in immune animals the IgM persisted for long periods. The last finding is thought to reflect non-sterile immunity.

CHAPTER NINE

STRAIN DIFFERENTIATION

INTRODUCTION

Claims on the existence of immunological differences between strains of Cytoecetes phagocytophila are based on cross-immunity trials, susceptible animals being inoculated with one strain and subsequently challenged with other strains. Differences were estimated on the basis of the degree of cross-protection between strains and the length of the incubation period, the degree and duration of thermal reaction, the magnitude and duration of parasitaemia (Tuomi, 1967[b]) and the degree of neutropaenia (Foster and Cameron, 1970[a]) caused by such strains. An assessment of the quantitative relationship between strains of C. phagocytophila was considered possible in the light of my findings with the CF test.

MATERIALS AND METHODS

Strains of C. phagocytophila

The Old Sourhope (OS) strain, first isolated from sheep by Foster and Cameron (1970[a]), the Sourhope R153 (SR153) strain isolated in 1976 from a Cheviot ewe on the same farm in the north of Scotland (Scott, 1979) and the Lephinmore-1 (L-1) strain isolated in 1978 from a Blackface lamb on a farm in the west of Scotland (Scott, 1979) were used. Stabilates of the strains were prepared from heparinised infected sheep blood and stored in the vapour phase of a liquid-nitrogen refrigerator at -114°C using 10 percent dimethyl sulphoxide as a cryoprotectant.

Experimental Sheep

Three adult sheep which were obtained from tick-free areas

were used as sources of antigen and sera. They were intravenously inoculated with one ml of a stabilate diluted ten-fold with phosphate buffered saline at pH 7.2.

Antigen

Infected sheep were bled at the peak period of parasitaemia in heparinised sterile containers. The infected blood was mixed with three volumes of medium 199 with HEPES buffer supplemented with ten percent foetal calf serum and 100 IU of penicillin per ml, and incubated at 37°C for 24 hours under continuous magnetic stirring. This resulted in the increase in the number of infected cells and improvement of antigen yields (Chapter Six). The red blood cells were lysed with 0.83 percent ammonium chloride solution. The cells were washed with phosphate buffered saline three times and resuspended in distilled water to give a concentration of at least 10^7 infected cells per ml. The cell suspensions were distributed in aliquots of two ml and treated with ultrasonic vibration (MSE Ltd.) for 90 seconds. They were titrated against standard homologous immune serum and stored at -114°C until required. The dilution of antigen which resulted in optimum fixation of complement in the presence of homologous immune serum in a chequerboard titration, was used for the subsequent tests.

Sera

Sera were collected from each sheep before inoculation with C. phagocytophila and two and three weeks later, when the antibody levels were expected to be high (Chapter Five) and stored at -20°C until use. All sera were treated with complement and inactivated at

56°C for 30 minutes to eliminate anticomplementary activities.

Tube Test

All tests were carried out in plastic conical centrifuge tubes (Sterilin Ltd.). The complement was freeze-dried normal guinea pig serum (Wellcome Reagents Ltd.). It was titrated and the dilution giving 50 percent haemolysis of 1.5 percent sensitised sheep erythrocytes (SSRBC) was taken as one unit of complement. A series of dilutions containing two, four, six, eight and ten units of complement were prepared in aliquots of 0.5 ml in an ice bath.

Three series of two-fold dilutions of inactivated sera from each of the three strains were prepared. An equal amount of homologous antigen was added to one of the series of dilutions. Each of the other two series were mixed with equal volumes of one of the heterologous antigens. Then 0.5 ml of the antigen-serum mixtures were added to each of the series of dilutions of complement. Appropriate controls for antigens, sera, complement and SSRBC were also set up. All tests were set in triplicate. The 1660 tubes were left at 4°C overnight.

The haemolytic system and the tubes were incubated at 37°C for 30 minutes before 0.25 ml of SSRBC were added to all the tubes. The tubes were incubated with agitation for 45 minutes at 37°C. The test was read after two hours at 4°C and five minutes centrifugation at 600 g at 4°C. The optical density of the supernatant was determined with a spectrophotometer (SPG-200 Pye Unicam) at 541 nm to estimate the percentage of haemolysis of SSRBC. The

absorbance was set at zero using cell controls. The specific fixation of each antigen-antibody reaction was estimated as the difference between the fixation of the reaction and the fixation displayed by the corresponding antigen and serum controls. The dilution of sera and the units of complement fixed were plotted and the titre of serum was expressed as the highest dilution which fixed at least two units of complement in the presence of two units of antigen.

Microplate Test

The microplate tests were performed in plastic plates containing U-shaped 96 wells (Sterilin Ltd.). The CF test was carried out as described in Chapter Five. The titre of the antiserum was determined by chequer-board titration with homologus and heterologous antigens. The titre of each serum against antigens of all strains tested was the highest dilution of serum which fixed 75 percent of two units of complement by visual interpretation, with an optimum concentration of antigen.

Calculation of Strain Differences

Calculation of relatedness and dominance between strains. The relation between two strains was expressed as the square root of the product of their cross-fixation ratios as suggested by Archetti and Horsfall (1950). The antigenic relationship was then determined with the formula :

$$R = \sqrt{r_1 \times r_2}$$

where $r_1 = \frac{Xy}{Xx}$; $r_2 = \frac{Yx}{Yy}$

and,

where Xy = titre of serum X with heterologous antigen y

Xx = titre of serum X with homologous antigen x

Yx = titre of serum Y with heterologous antigen x

Yy = titre of serum Y with homologous antigen y

The relation between two strains was also determined from the amount of complement fixed by various dilutions of sera reacting with homologous and heterologous antigen (Davie, 1964). In this case the R^1 values were derived from the ratios (R^1) of the amounts of complement fixed by heterologous reactions and homologous reactions.

The degree of dominance (D) of one strain over another strain was expressed as the square root of the R values of the two strains (Pereira, 1977).

Thus, $$D = \sqrt{\frac{R_1}{R_2}}$$

where $R_1 = \frac{Xy}{Xx}$ and $R_2 = \frac{Yx}{Yy}$

In the above example D values greater or smaller than 1.0 were considered to indicate dominance of strain X over strain Y or strain Y over strain X respectively. Similarly D^1 values were obtained from the square roots of the ratios of R^1 values of two strains derived from the amount of complement fixed.

RESULTS

Tube Test

Relatedness. The titres of all three strains were higher with homologus antigen than with heterologous antigen (Figures 22-24, Table 44).

The R values estimated from the ratios of the titres of each serum with heterologous and homologous antigen are shown in Table 45. The R values indicated differences among the three strains. The difference between OS and SR153 strains were less marked than the difference between OS and L-1 strains or between SR153 and L-1 strains. The R^1 values estimated from the amount of complement fixed by dilution of sera reacting with heterologous and homologous antigens are shown in Table 46. At high concentrations of sera the amount of complement fixed by homologus reactions was not always significantly higher than the amount of complement fixed by heterologous reactions. However, when sera were diluted beyond 2^{-5} the amount of complement fixed by homologous reactions was more than the amount fixed by heterologous reaction.

Dominance. The D values estimated from the serum titres are shown in Table 47. The OS strain was dominant over the L-1 strain (D = 1.26); the SR153 strain was also dominant over the L-1 strain (D = 1.43) but there was very little dominance between the SR153 and OS strains (D = 0.93). When D^1 values were estimated from the amounts of complement fixed (Table 48) the dominance of OS and SR153 strains over the L-1 strain became apparent only at high dilutions of serum. No dominance between the OS and SR153 strain was apparent at any dilution.

Microplate Tests

Relatedness. When sera were titrated with dilutions of homologous and heterologous antigens in microplates with constant amounts of complement, typical slopes of high titres with optimal

concentrations of antigen and lower titres with low concentrations of antigen were observed (Figures 25 to 27). The optimal dilutions of antigen were similar whether tested against homologus or heterologous sera. The results of chequer-board titration revealed that all sera tested had their highest titres when tested against the homologous antigens. The titres of OS and SR153 were closely similar, the differences being two-fold dilutions only but the differences between OS and L-1 and between SR153 and L-1 were very marked (Figures 25 to 27). The analysis of their cross-fixation ratios (R values) revealed that there is a very close relationship between the OS and SR153 strains but the L-1 strain markedly differed from both strains (Table 45).

Dominance. The L-1 strain appeared to be slightly dominant over both OS and SR153 strains but there was no dominance between the OS and the SR153 strains (Table 47).

Figure 22 Amounts of complement fixed by dilutions of anti-OS serum against homologous and heterologous antigens

A - against OS antigen

B - against SR153 antigen

C - against L-1 antigen

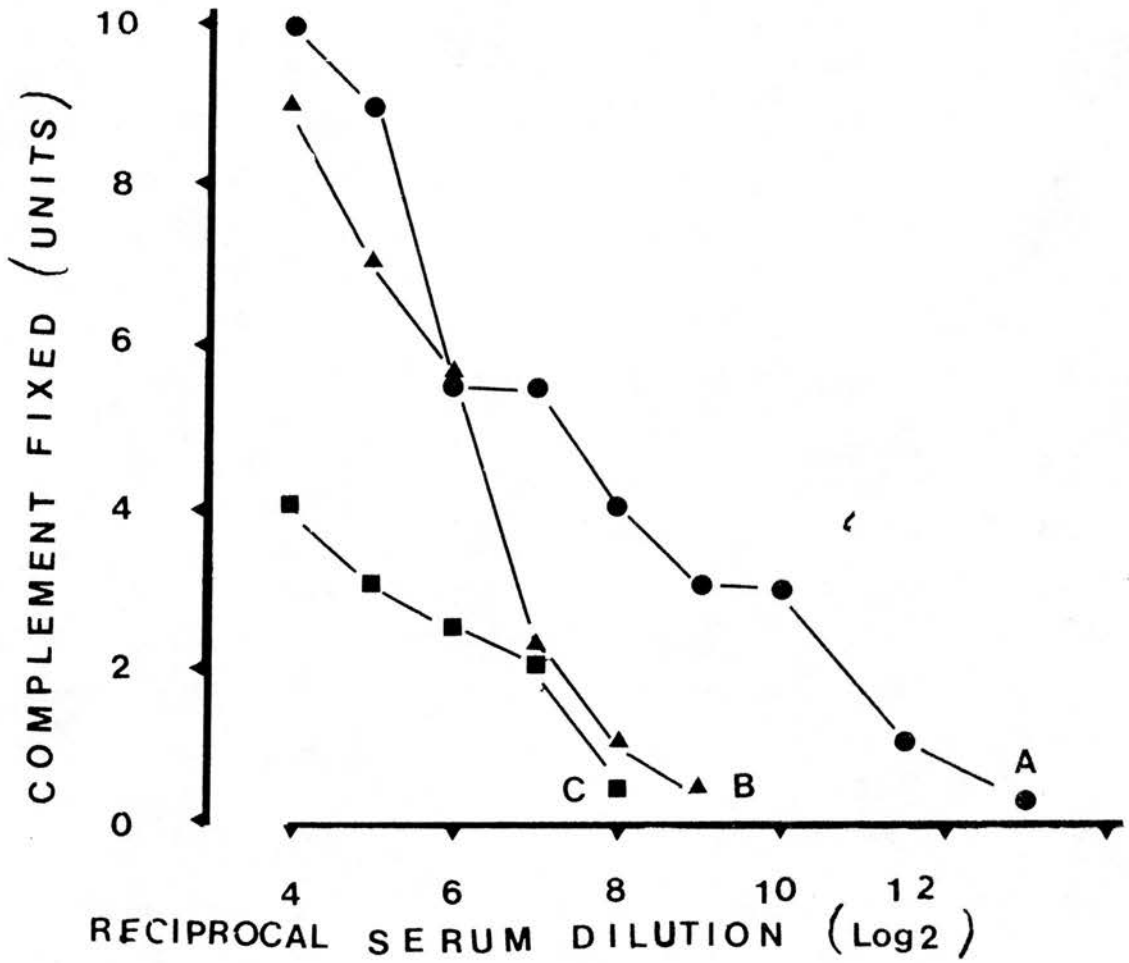

COMPLEMENT FIXED (UNITS)
10
8
6
4
2
0
A
B
C
4
6
8
10
12
RECIPROCAL SERUM DILUTION (Log2)

Figure 23 Amounts of complement fixed by anti-SR153 serum against homologous and heterologous antigens

A - against OS antigen

B - against SR153 antigen

C - against L-1 antigen

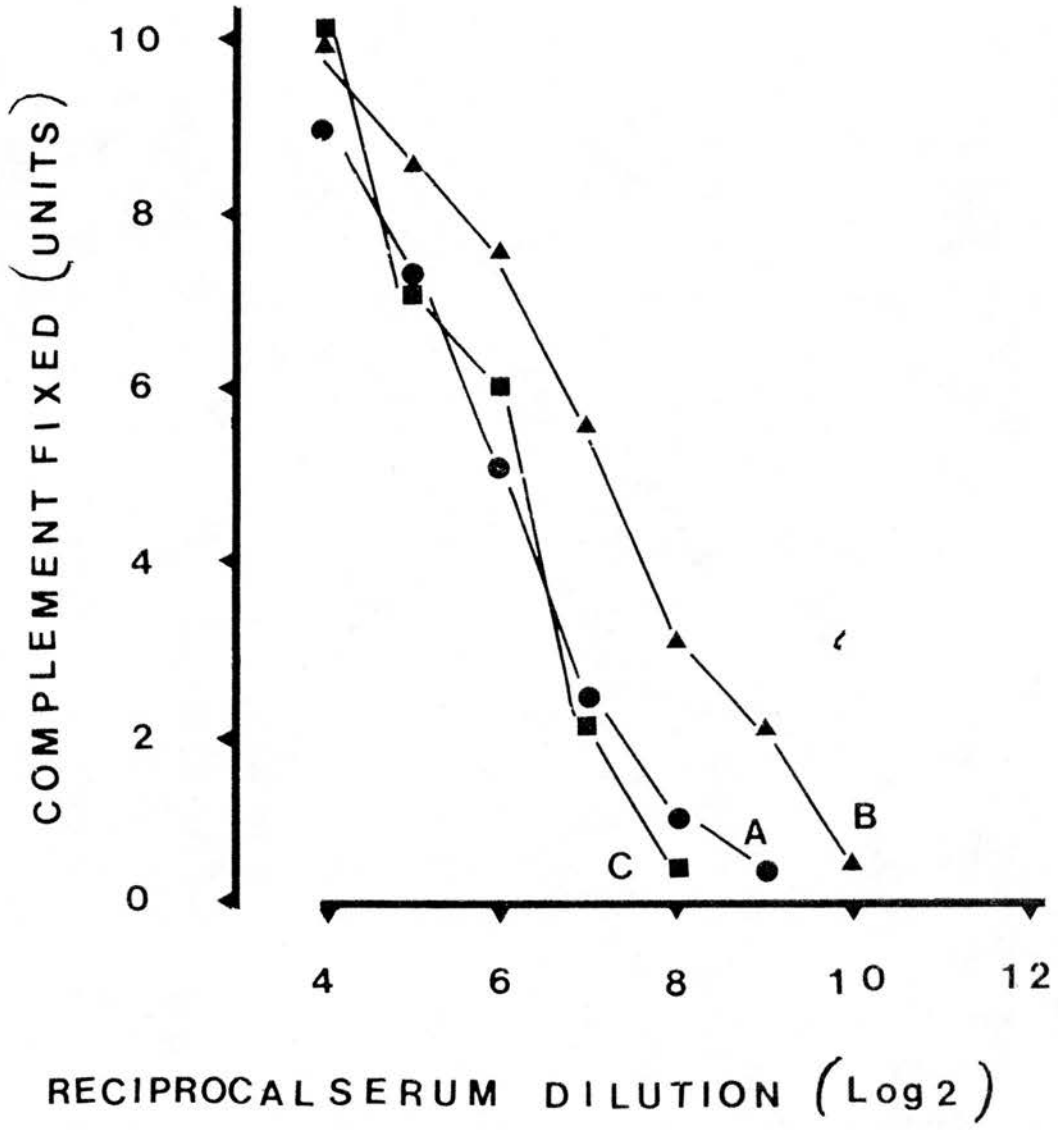

10
8
6
4
2
0
COMPLEMENT FIXED (UNITS)
A
B
C
4
6
8
10
12
RECIPROCAL SERUM DILUTION (Log 2)

Figure 24 Amounts of complement fixed by anti L-1 serum against homolgous and heterologous antigens

A - against OS antigen

B - against SR153 antigen

C - against L-1 antigen

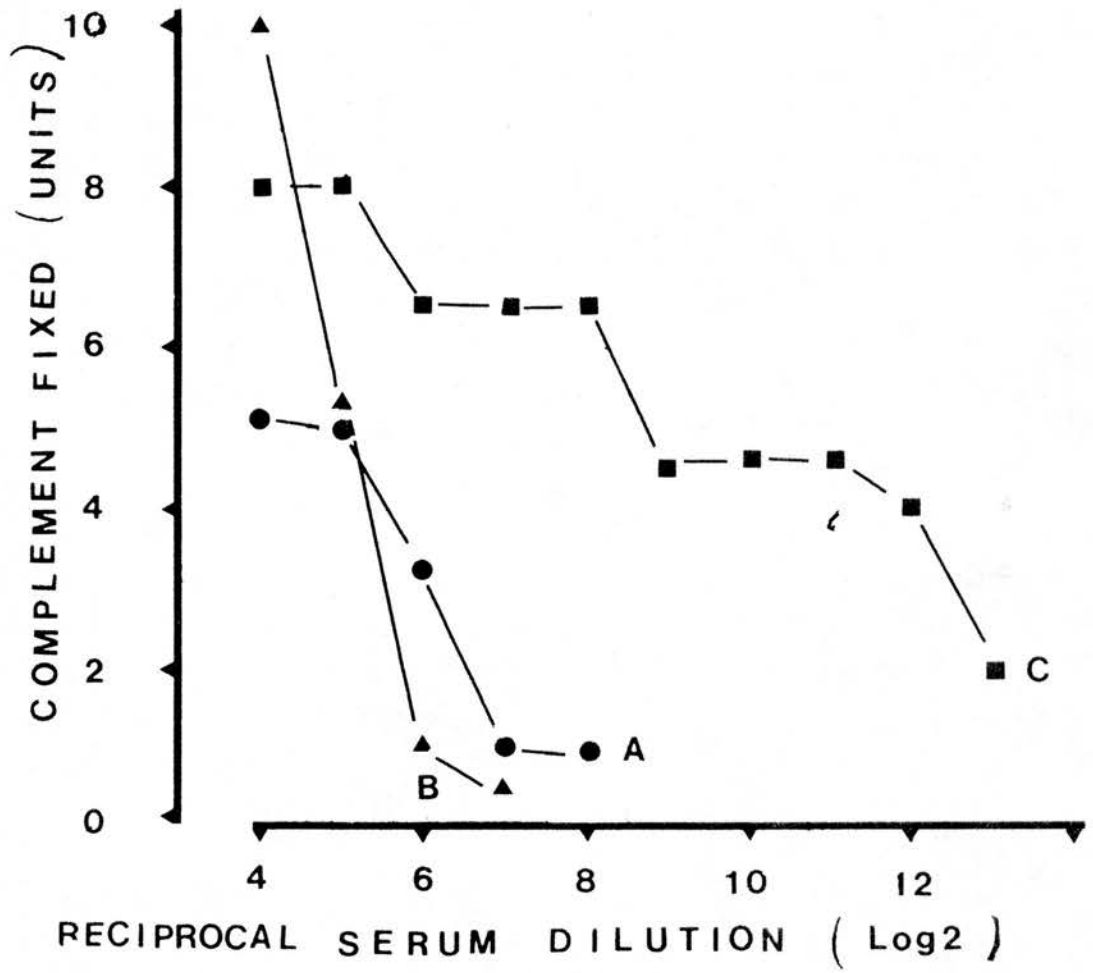

COMPLEMENT FIXED (UNITS)
10
8
6
4
2
0
A
B
C
4
6
8
10
12
RECIPROCAL SERUM DILUTION (Log2)

Figure 25 Chequer-board titration of anti-OS serum against homologous and heterologous antigens (microplate)

A -against OS antigen

B - against SR153 antigen

C - against L-1 antigen

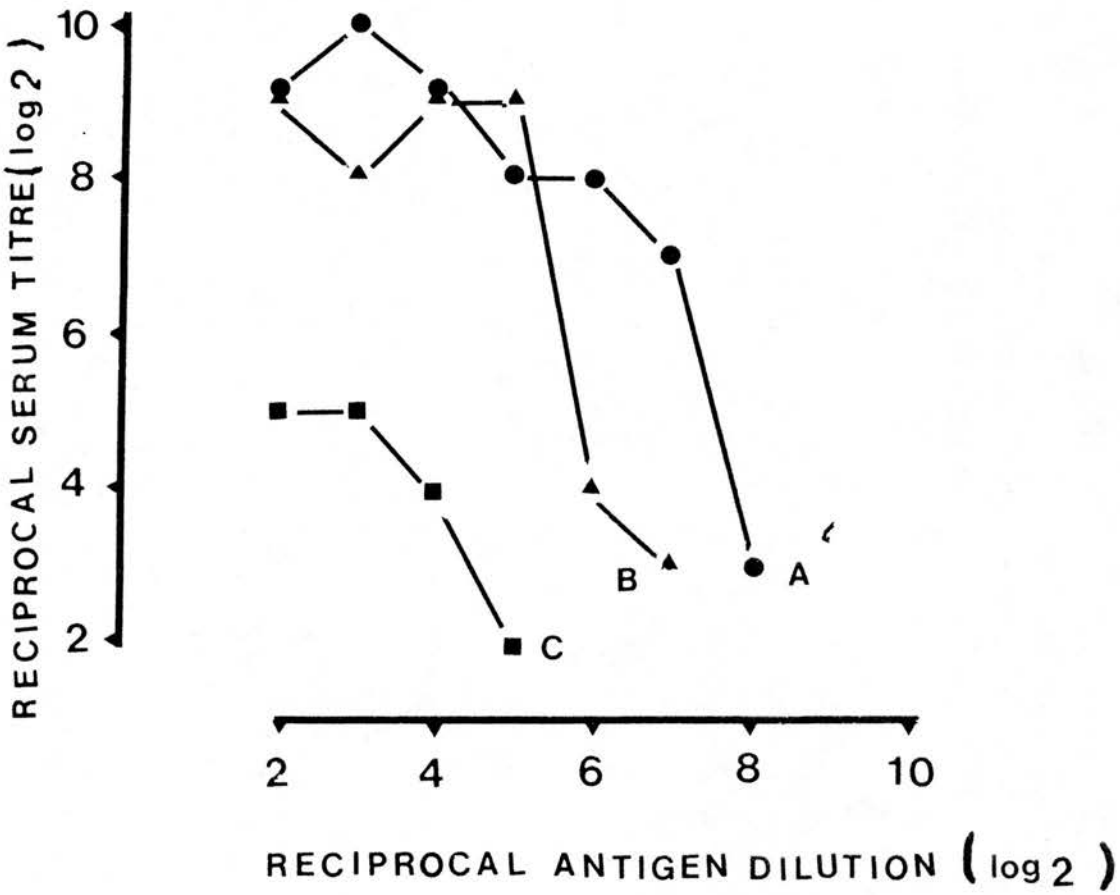

RECIPROCAL SERUM TITRE(log 2)
10
8
6
4
2
A
B
C
2
4
6
8
10
RECIPROCAL ANTIGEN DILUTION (log 2)

Figure 26 Chequer-board titration of anti-SR153 serum against homologous and heterologous antigens (microplate)

A - against OS antigen

B - against SR153 antigen

C - against L-1 antigen

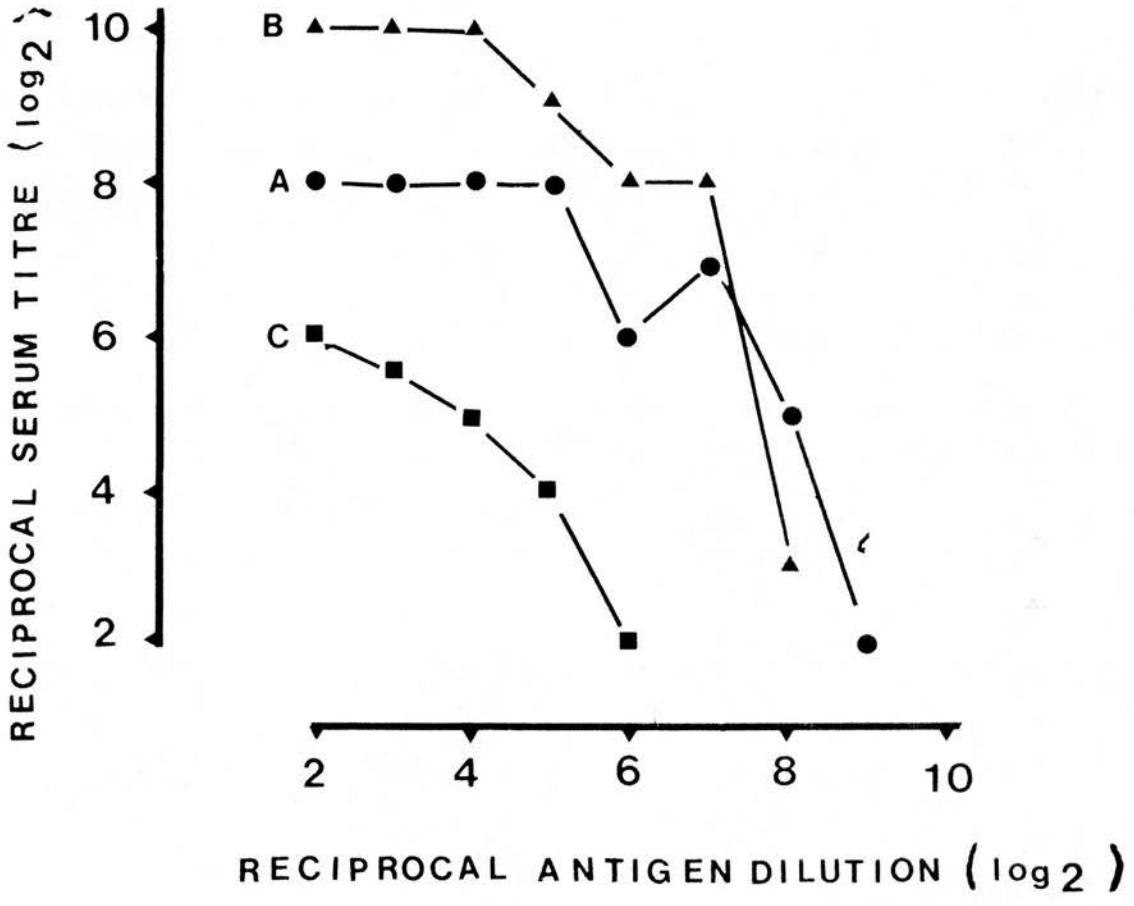

RECIPROCAL SERUM TITRE (log2)
10
8
6
4
2
B
A
C
2
4
6
8
10
RECIPROCAL ANTIGEN DILUTION (log2)

Figure 27 Chequer-board titration of anti-L-1 serum against homologous and heterologous antigens (microplate)

A - against OS antigen

B - against SR153 antigen

C - against L-1 antigen

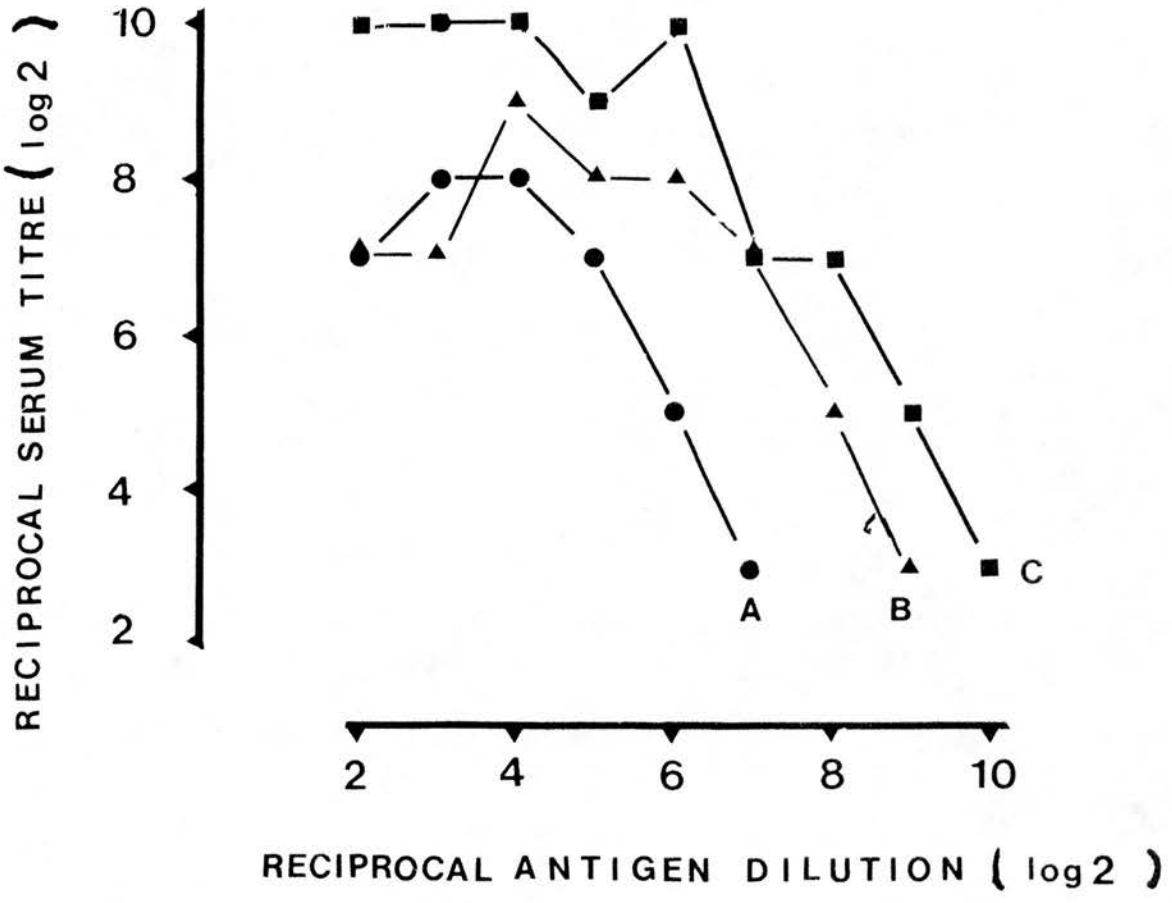

RECIPROCAL SERUM TITRE (log 2)
10
8
6
4
2
A
B
C
2
4
6
8
10
RECIPROCAL ANTIGEN DILUTION (log 2)

Table 44 Reciprocal CF antibody titres ($\log_2$) of sera against homologous and heterologous antigens

	Serum	Antigen OS	Antigen SR153	Antigen L-1
Tube test	OS	12	8	7
	SR153	7	9	7
	L-1	6	5	13
Microplate test	OS	10	9	5
	SR153	8	10	6
	L-1	8	9	10

Table 45 R values estimated from the titres of sera

	Strain	OS	SR153	L-1
Tube test	OS	1.00		
	SR153	0.72	1.00	
	L-1	0.52	0.55	1.00
Microplate test	OS	1.00		
	SR153	0.85	1.00	
	L-1	0.63	0.73	1.00

Table 46 R^1 values estimated from the amount of complement fixed by homologous and heterologous reactions

Strain	OS	SR153	L-1	Reciprocal dilution (log_2)
OS	1.00			4
SR153	0.90	1.00		4
L-1	0.52	1.00	1.00	4
OS	1.00			5
SR153	0.80	1.00		5
L-1	0.46	0.78	1.00	5
OS	1.00			6
SR153	0.57	1.00		6
L-1	0.50	0.35	1.00	6
OS	1.00			7
SR153	0.45	1.00		7
L-1	0.24	0.23	1.00	7

Table 47 D values estimated from the titres of sera against homologous and heterologous antigens

	$r^{OS}/_{r\,L-1}$	$r^{OS}/_{r\,SR153}$	$r^{SR153}/_{r\,L-1}$
Tube test	1.26	0.93	1.43
Microplate test	0.79	1.06	0.91

Table 48 D^1 values estimated from the amount of complement fixed by homologous and heterologous reactions

Reciprocal serum dilution (log_2)	r^1 OS/r^1 L-1	r^1 OS/r^1 SR153	r^1 SR153/r^1 L-1
4	0.76	1.00	0.89
5	0.73	0.94	1.09
6	1.18	1.17	2.28
7	1.60	1.00	2.26

DISCUSSION

My findings revealed that the antigenic relationship among ovine strains was very strong but I also found that appreciable differences could be demonstrated quantitatively. All the sera had higher CF antibody titres with homologous antigen than with heterologous antigens. At higher dilutions all the sera fixed more complement with homologous antigen than with heterologous antigens. The results suggested that there was a close relationship between the OS and SR153 strains while the L-1 strain markedly differed from both OS and SR153 strains. Differences based on the ratios of antibody titres with heterologous antigen and homologous antigen have been used in the classification of viruses, especially for sub-typing foot-and-mouth disease viruses; strains with R or R^1 values higher than 0.70 being regarded as belonging to the same sub-type while those showing R values between 0.32 to 0.70 were regarded as different sub-types. However, Pereira (1977) pointed out that strict adherence to such criteria of arbitrary levels of cross-reactivity fails to appreciate other factors such as asymmetrical relationship or dominance of one strain over another. The R and R^1 values obtained in this study were low enough to suggest that at least the L-1 strain could be regarded as a different sub-type.

The findings in this study suggested that the differences between strains of C. phagocytophila were better demonstrated by comparing serum titres with homologous and heterologous antigens. The tube test which is based on colorimetric assessment of complement fixation was found to be more sensitive than the microplate technique.

The relatively less sensitive nature of the microplate test may be due to the semi-quantitative nature of visual interpretation and to the constant use of high concentrations of complement. This is supported by the fact that in the tube test no significant differences between complement fixed by homologous and heterologous reactions were observed at high concentrations of sera. However, at higher dilutions of sera, more complement was fixed by homologous reactions than by heterologous reactions. These findings suggest that titrations of sera against complement with optimum concentrations of homologous and heterologous antigen will demonstrate differences between strains quantitatively.

Both the tube test and the microplate test were sensitive for differentiating strains but the former was more accurate, relatedness and dominance being easily detected at higher dilutions of sera.

CHAPTER TEN

PRELIMINARY STUDIES ON THE CELL-MEDIATED IMMUNE RESPONSES TO C. PHAGOCYTOPHILA

INTRODUCTION

Mackaness (1969) demonstrated that cell-mediated immunity (CMI) is an important mechanism in the destruction of intracellular facultative organisms and the establishment of an immune state. Macrophages play an important role in this process. The process of their activation involves immunological events which do not depend upon circulating antibodies (Mackaness, 1964). The activation of the macrophages involves the production of mediator substances by immunologically committed lymphocytes. The mediator substances known as lymphokines (Dumonde, Wolestencroft, Panayi, Mathew, Morley and Howson, 1969) have properties of inhibiting the migration of macrophages, evoking the increased mitosis (transformation) of lymphocytes and initiating the exudation of cells, when injected intradermally.

In vitro methods of assessing CMI have been appearing with increasing frequency over the last few years. Macrophage migration inhibition (MI) and lymphocyte transformation are now widely accepted as in vitro reflections of delayed type hypersensitivity reactions in guinea pigs (Bennet and Bloom, 1968; Thor, Jureziz, Veach, Miller and Droy, 1968). Søborg and Benedixen (1967) introduced an important improvement to the then commonly used macrophage migration inhibition test. Instead of using guinea pig peritoneal macrophages they used white blood cells from brucella-infected persons. They reported that there was an inhibition of the migration of leucocytes (LMI) of brucella-infected persons when the leucocytes were cultured in the presence of brucella

antigen. Since then many workers have reported similar results in experiments involving various antigens and animals.

Recently the LMI test has found use in the study of CMI response to rickettsial organisms. Buening (1973) and Carson and his colleagues (1977) reported its successful use in the study of CMI response of animals infected with anaplasmosis and Nyindo and his colleagues (1980) claimed that LMI can serve as a reliable measure of CMI in animals infected with Ehrlichia canis. The present work was aimed at investigating the CMI response of sheep infected with C. phagocytophila.

MATERIALS AND METHODS

Leucocyte Migration Inhibition Test

Culture medium. RPMI 1640 with HEPES buffer was supplemented with 20 percent foetal calf serum and 100 IU penicillin per ml before use. When leucocytes were cultured for the isolation of lymphokines, no serum was added.

Preparation of glassware. All bottles and Pasteur pipettes used in the test were siliconised by immersing them in dimethyldichlorosilane solution in about two percent 1, 1, 1, trichloroethane (British Drug House). They were then rinsed in distilled water, dried and autoclaved before use.

Preparation of antigens. The antigens were prepared as described in Chapter Five.

Direct leucocyte migration inhibition test. Ten ml of blood

was collected into heparinized sterile containers. An equal amount of 0.83 percent ammonium chloride solution was added to lyse the erythrocyte in siliconized containers. As soon as the erythrocytes were lysed, two volumes of HBSS were added and the cells centrifuged at 300 *g* for ten minutes. After one more washing they were resuspended in RPMI 1640 medium to make a final concentration of 10^7 cells per ml. The cells were aspirated into capillary tubes (Hawksley and Son Ltd.) and then one end sealed by pressing into Crystaseal Wax (Hawksley and Son Ltd.). They were then centrifuged at 2000 *g* for five seconds (Maeda, 1980) in a Microhaematocrit Centrifuge (Hawksley and Son Ltd.). The capillary tubes were cut at the cell-fluid interface and firmly attached to migration chambers with a blob of silicone grease. Then growth medium RPMI 1640 was added. Antigens and control extracts from normal leucocytes were added to appropriate chambers. Then the edges of the chambers were greased with silicone and sealed with heat-treated square coverslips and incubated at $37^{\circ}C$ in an atmosphere of five percent carbon dioxide for 24 hours. The image of the tube and the area of migrating cells were viewed by a photoenlarger (De Vere 54 Varion) and immediately projected onto graph paper. The outline of the cell migration area was traced out and the area of migration determined by counting the mm squares within the outline.

Partial characterisation of the leucocyte migration inhibitory factor (LMIF). The LMIF was characterised partially according to the method of Bennet and Bloom (1968).

LMIF1. Blood was collected from infected sheep at the second day of parasitaemia and processed as described earlier. The harvested leucocytes were then cultured in medium RPMI 1640 with HEPES buffer and 100 IU of penicillin per ml but without serum supplement in 75 cm^2 flasks as described in Chapter Three. After 24 hours the supernatant was harvested and centrifuged at 600 g for ten minutes. The supernatant was then dialysed against large volumes of normal saline for 24 hours and concentrated by dialysis against polyethylene glycol 2000 to approximately five percent of the original volume. The material was labelled LMIF1 and stored at $-20^{\circ}C$ until use.

LMIF 2, LMIF 2A, and LMIF C. Leucocytes were prepared from blood of infected sheep three weeks after the cessation of clinical disease and parasitaemia and cultured as described earlier with or without the addition of C. phagocytophila antigen. The supernatant from cells cultured without antigen was labelled LMIF 2 and the one from cells cultured with antigen was labelled LMIF 2A. Leucocytes from normal, uninfected sheep were also cultured with antigen and the supernatant labelled LMIF C. All were dialysed against normal saline, concentrated as described earlier and stored at $-20^{\circ}C$ until required.

Indirect leucocyte migration inhibition test. The tests were carried out to study the effects of LMIF on the migration of leucocytes of normal, uninfected sheep and the peritoneal exudate (PE) cells of guinea pigs. Leucocytes were prepared from nine sheep as described earlier. The capillaries were then put into

migration chambers containing LMIF 1, LMIF 2, LMIF 2A and LMIF C. The images were projected and the migration areas compared.

Preparation of guinea pig PE cells. To produce guinea pig PE cells four healthy adult guinea pigs were injected intraperitoneally with 20 ml of liquid paraffin and three days later the peritoneal cavities were irrigated with about 100 ml of HBSS. The cells were washed twice with HBSS and resuspended in RPMI 1640 medium to give 10^6 viable cells per ml. They were aspirated into capillary tubes as described earlier and set in migration chambers containing LMIF 1, LMIF 2, LMIF 2A and LMIF C.

Delayed Type Hypersensitivity (DTH) Test

Antigens were prepared as described in Chapter Five. Control extract was also prepared from leucocytes of normal sheep. A group of eight sheep were then inoculated with 0.1 ml of antigen on the medial aspect of the right thigh and the control extract was injected intradermally on the left thigh. The sites were inspected for induration after 24, 48 and 72 hours. Subsequently the same sheep were infected with C. phagocytophila and after 21 days the test repeated.

Passive Transfer of Sensitised Peripheral Leucocytes

Attempts to transfer sensitised cells to normal, uninfected sheep were made by injecting them with peripheral mononuclear cells of sheep which had resisted challenge and which had shown strong CMI response as measured by the LMI test. Five sheep which had experienced primary infection were challenged after four months.

None of them reacted clinically and parasitaemia was not detected. Three weeks later each sheep was bled into heparinized containers and the buffy coat separated by centrifugation. The mononuclear cells were separated by density gradient centrifugation using Lymphoprep (Nygaard, Oslo) as described in Chapter Six. The mononuclear cells were washed three times in RPMI 1640 medium and the viability of the cells estimated by trypan blue exclusion technique (Hanks and Wallace, 1958). Then ten ml of a 10^{10} per ml suspension in RPMI 1640 injected into susceptible sheep intravenously. To check for the possibility of the transfer of cell-associated C. phagocytophila, the newly injected sheep were kept under observation for possible TBF reaction for seven days. Those which did not react were then challenged with C. phagocytophila. The donor sheep whose mononuclear cells did not provoke TBF, were again bled and the mononuclear cells separated and two new susceptible sheep injected with a ten ml of a 10^{10} cell suspension. Twenty hours later they were inoculated with C. phagocytophila and kept under clinical observation.

Experiment Design

The direct LMI test was used to assess the onset and duration of CMI in sheep infected with C. phagocytophila and the indirect test used to study the specificity of the LMIF. The following groups were used:

Groups 1 and 2 consisted of nine control sheep injected with normal saline and eight sheep infected with C. phagocytophila. The sheep were bled every three to four days for a period of eight

weeks. The leucocytes were prepared as described earlier and cultured in migration plates with and without antigen.

Groups 3 and 4 consisted of eight normal sheep which were injected with normal saline and eight sheep infected with C. phagocytophila. They were bled every 24 hours starting from the day before inoculation until the onset of parasitaemia in the infected sheep. Leucocytes were cultured in migration plates in the presence and absence of antigen.

Group 5. Two sheep which had experienced primary infection and had shown strong CMI response as measured by the direct LMI were challenged with C. phagocytophila four months after primary infection and leucocytes were cultured with or without antigen in migration plates.

Group 6 consisted of nine sheep which were not infected with TBF. Leucocytes were cultured with or without LMIF.

Analysis of Data

All migration inhibition tests were set up in triplicates. The effects of antigen on the migration of leucocytes were assessed either by analysis of pair-difference using the Student's t-test or by using the migration index (MI).

$$MI = \frac{\text{Area of Migration of Leucocytes without Antigen}}{\text{Area of Migration of Leucocytes with Antigen}} \times 100$$

For the comparison of two independent groups the MIs were analysed by Student's t-test. For the comparison of the migration of infected cells with the migration of normal, uninfected cells pair differences of migration areas before and after inoculation with

C. phagocytophila were analysed by Student's t-test or by analysing the means and standard deviations of the two groups by analysis of variance.

To analyse the effects of LMIF on normal sheep leucocytes or guinea pig PE cells, either pair differences or the areas of migration with and without LMIF were analysed with Student's t-test or the migration indeces were calculated as follows:

$$MI = \frac{\text{Areas of Migration of Cells with LMIF}}{\text{Areas of Migration of Cells without LMIF}} \times 100$$

RESULTS

Leucocyte Migration Inhibition Test

The direct leucocyte migration inhibition test revealed that infections with C. phagocytophila were quickly followed by a CMI response.

Groups 1 and 2. The migration areas of leucocytes obtained from sheep before and after injection with normal saline were not significantly different when cultured without antigen (Table 49, Appendix Table 52). When antigens were added to the migration chambers, the results were the same (Table 50, Appendix Tables 52 and 53).

The migration areas of leucocytes obtained from the eight sheep which were infected with C. phagocytophila were significantly reduced from the areas of migration before infection when they were cultured without antigen (Table 51, Appendix Table 54). When the leucocytes were cultured with antigen their migration areas were significantly smaller than the areas of migration without

antigen (Table 52, Appendix Tables 54 and 55). The reductions attributable to the addition of antigen became more pronounced after 24 to 28 days post-inoculation.

Comparisons of the MI of leucocytes from infected and from uninfected sheep revealed that they differed significantly at least three weeks after inoculation (Figure 28, Table 53).

Groups 3 and 4. The migration areas of leucocytes obtained before and after injection with normal saline were compared and significant differences were not observed when they were cultured without antigen (Table 54, Appendix Table 56). There were no significant differences between the migration areas of those cultured with antigen and those cultured without antigen (Table 55, Appendix Tables 56 and 57).

The migration areas of leucocytes obtained before infection with C. phagocytophila and the migration areas obtained after infection were compared. There were no statistically significant changes in the migration areas of leucocytes obtained 24 and 48 hours after inoculation but the migration areas of leucocytes obtained on the third day post-inoculation were significantly smaller than the migration areas of those obtained before inoculation (Table 56, Appendix Table 58). The reduction of areas of migration coincided with onset of parasitaemia (Figures 20 and 30, Table 56). The reductions in migration areas of leucocytes obtained from infected sheep were not due to the addition of antigen (Table 57, Appendix Tables 58 and 59).

Group 5. The leucocytes obtained from two sheep which resisted

challenge inoculation showed reduced migration areas when cultured with antigen, the migration indices being 48 and 71 percent respectively five days after challenge.

Group 6. Leucocyte migration inhibitory factors were successfully isolated from leucocytes of infected sheep obtained during the peak period of parasitaemia and cultured without antigen (LMIF 1) and from leucocytes obtained from sheep which had recovered from clinical disease and by culturing them with and without antigen (LMIF 2A and LMIF 2 respectively). The LMIF reduced the migration areas of leucocytes obtained from nine uninfected sheep. The reductions caused by LMIF 1 were statistically significant ($t_6 = 3.72$, $p < 0.01$) the MI being 21 ± 23 percent. The reductions due to LMIF 2 were not statistically significant ($t_8 = 0.64$, $p > 0.060$) the MI being 95 ± 15 percent but the reductions due to LMIF 2A were significant ($t_8 = 4.64$, $p < 0.010$) the MI being 30 ± 13 percent (Appendix Table 60).

The LMIF prepared from leucocytes of normal, uninfected sheep (LMIF C) had no effect ($t_6 = 0.28$, $p > 0.80$) the MI being 100 ±12 percent.

The effects of LMIF on guinea pig PE cells were inconclusive because the PE cells' migration was very poor. Both injections of liquid paraffin or RPMI 1640 with or without Freund's incomplete adjuvant did not produce enough viable cells and the migration areas were too small for comparisons to be made.

Delayed Type Hypersensitivity Reactions

None of the eight sheep tested developed detectable DTH

reactions to C. phagocytophila antigen before or after inoculation with C. phagocytophila.

Passive Transfer of Sensitised Lymphocytes

The attempts to transfer PBL from five sheep which resisted reinfection by TBF were unsuccessful, the main problem being the presence of C. phagocytophila in the inocula. "Mononuclear" cells of the four sheep produced TBF reactions four to seven days after being injected into susceptible sheep. Only one sheep was not apparently a carrier. The "mononuclear" cells from the last sheep did not cause TBF reaction when injected into susceptible sheep. Moreover, they did not confer protection to the susceptible sheep against challenge after 24 hours or seven days.

Figure 28 Mean migration indices of leucocytes of normal uninfected sheep (A) and sheep infected with *C. phagocytophila* (B)

A - Group 1

B - Group 2

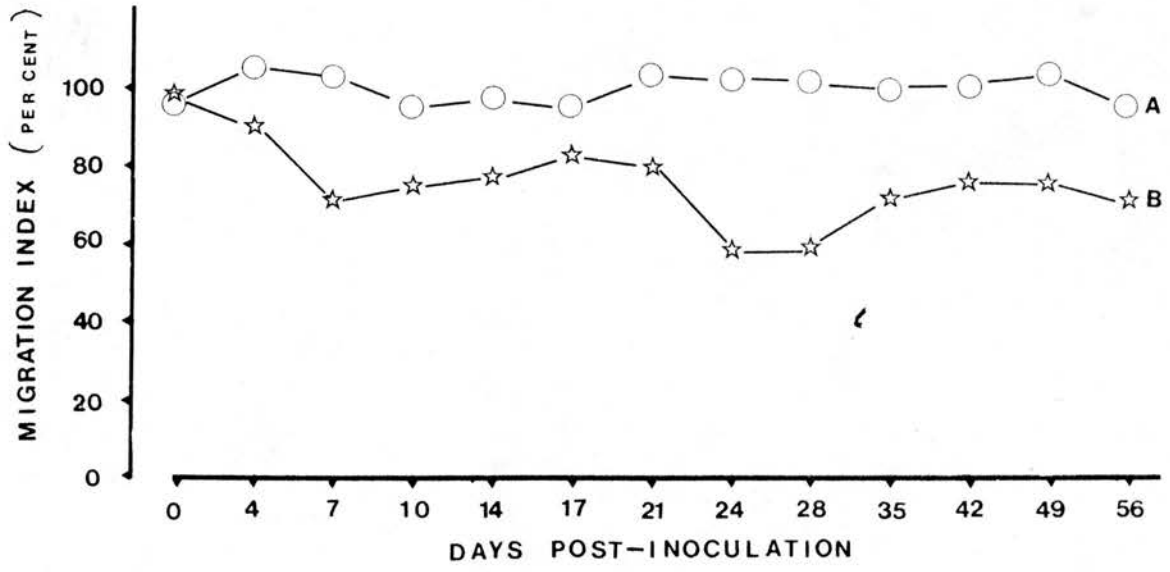

MIGRATION INDEX (PER CENT)
100
80
60
40
20
0
A
B
0
4
7
10
14
17
21
24
28
35
42
49
56
DAYS POST-INOCULATION

Figure 29 Parasitaemia (A) and areas of migration of leucocytes of sheep infected with *C. phagocytophila*

A - parasitaemia

B - migration areas

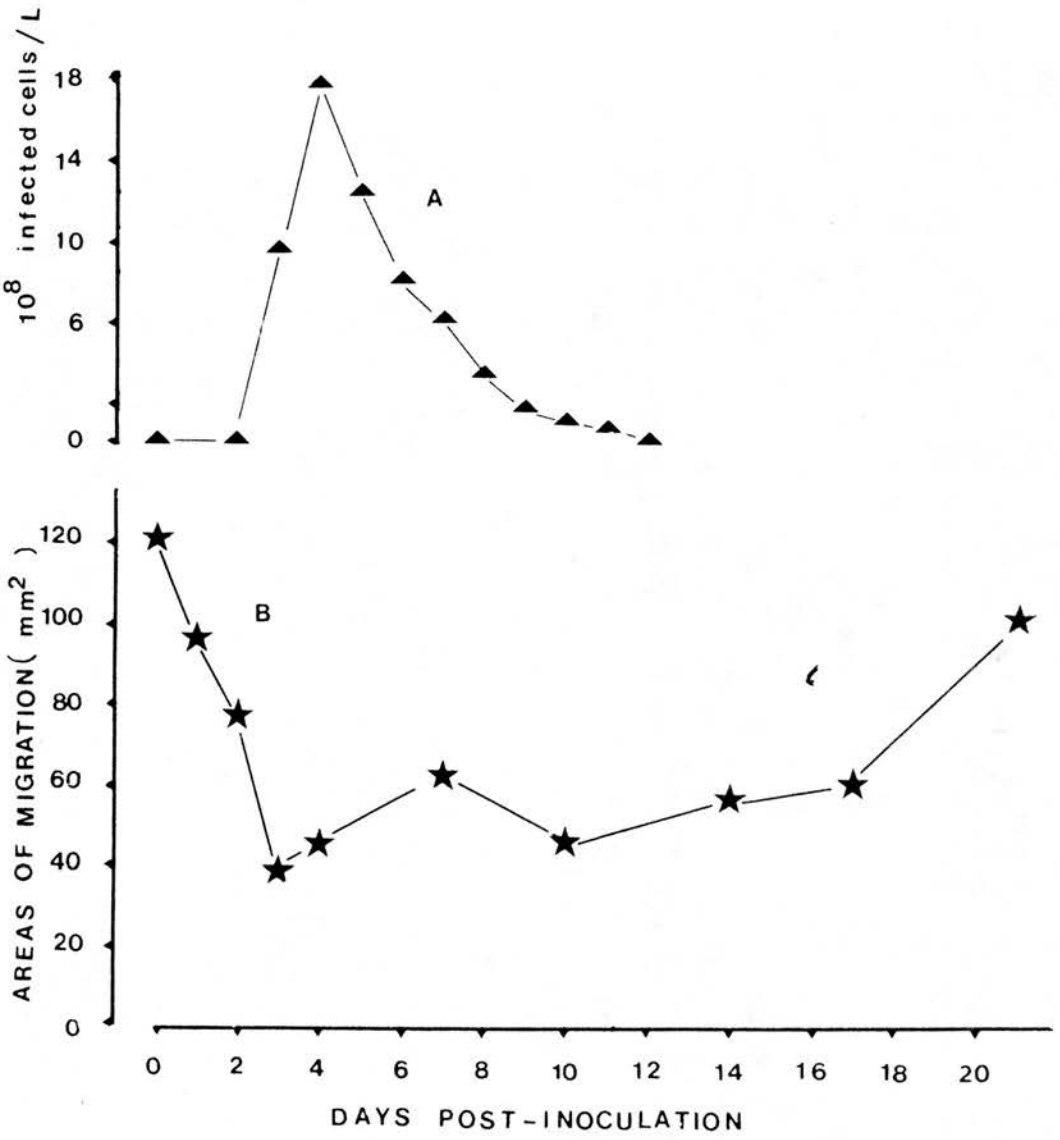
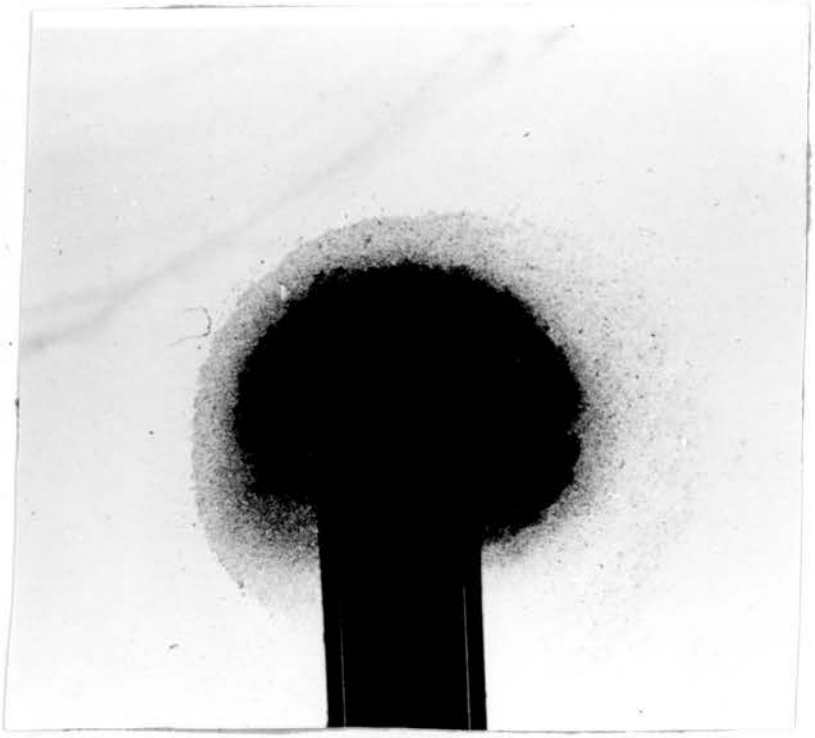

10^8 infected cells / L
18
14
10
6
0
A
AREAS OF MIGRATION (mm^2)
120
100
80
60
40
20
0
B
C
0
2
4
6
8
10
12
14
16
18
20
DAYS POST-INOCULATION

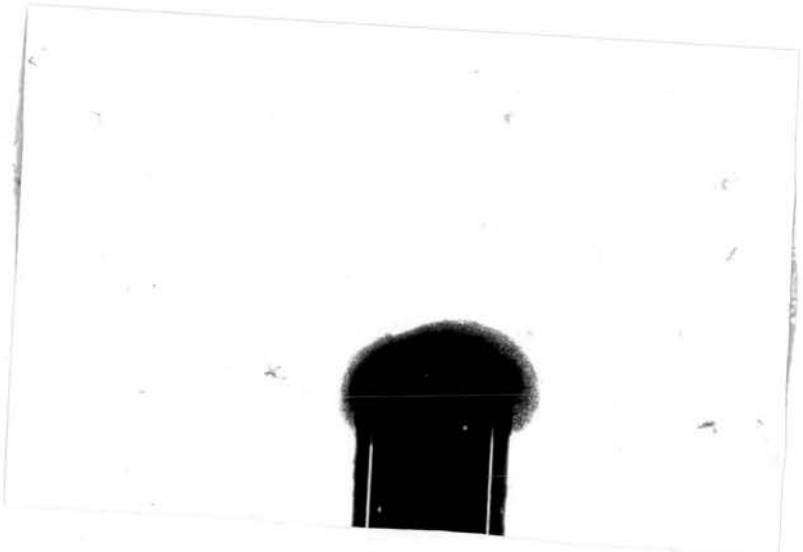
Figure 30 Migration of leucocytes of sheep infected with *C. phagocytophila*

A - Migration of leucocytes obtained before infection and cultured without antigen x 45

B - Migration of leucocytes obtained four days after inoculation with *C. phagocytophila* and cultured without antigen x 45



A



B

30

Table 49 Significance of the differences of the migration areas of leucocytes obtained from normal sheep before and after injection with normal saline and cultured without antigen (Day 0 - Day X)

Days post-injection	Degrees of freedom	Mean difference (mm^2)	e	t
4	7	-7.88	9.26	0.85
7	8	-18.33	14.18	1.29
10	6	- 0.71	6.63	0.11
14	8	- 3.22	14.98	0.21
17	6	- 0.14	21.11	0.01
21	6	-22.57	16.11	1.40
24	6	-18.86	23.86	0.79
28	8	- 8.44	17.75	0.48
35	8	10.44	10.39	1.00
42	8	- 4.00	20.21	0.20
49	8	-18.67	19.09	0.98
56	7	1.50	21.74	0.07

Table 50 Significance of the differences of migration areas of leucocytes of sheep injected with normal saline (Group 1) cultured with or without antigen

Days post injection	Degrees of freedom	Mean difference (mm^2)	e	t
0	7	- 4.56	3.72	1.23
4	8	2.38	5.50	0.43
7	6	1.22	3.78	0.32
10	8	- 3.71	2.69	1.38
14	6	- 5.44	2.94	1.85
17	6	- 2.86	2.72	1.05
21	6	-18.57	9.68	1.92
24	8	0.71	1.02	0.70
28	8	- 1.86	1.99	0.93
35	8	- 0.22	3.20	0.07
42	8	- 4.89	3.13	1.56
49	8	2.22	1.88	1.18
56	7	3.00	1.55	1.94

Table 51 Significance of the differences of migration areas of leucocytes obtained before and after infection with C. phagocytophila (Group 2) when cultured without antigen (Day 0 - Day X)

Days post-inoculation	Degrees of freedom	Mean difference (mm^2)	e	t
4	7	-86.25	27.58	3.13*
7	7	-82.50	22.59	3.65**
10	7	-92.50	27.71	3.34*
14	7	-89.88	24.65	3.65**
17	7	-75.38	23.77	3.17*
21	7	-34.50	13.41	2.57*
24	7	-67.38	30.33	2.22
28	7	-62.63	33.94	1.85
35	7	-55.13	30.19	1.83
42	7	-58.25	34.46	1.69
49	7	-70.38	31.43	2.24
56	5	-21.50	37.84	0.57

** $P < 0.010$; * $P < 0.050$

Table 52 Significance of the differences of migration areas of leucocytes obtained from infected sheep and cultured with or without antigen

Days post-inoculation	Degrees of freedom	Mean difference (mm^2)	e	t
0	7	0.38	4.23	0.09
4	7	7.63	6.01	1.27
7	7	18.25	5.37	3.40*
10	7	12.38	3.11	3.98**
14	7	- 6.25	5.50	1.14
17	7	-17.63	6.11	2.89*
21	7	-25.25	9.20	2.74*
24	7	-31.88	7.12	4.48**
28	7	-33.13	5.97	5.55***
35	7	-23.88	6.77	3.53**
42	7	-16.75	7.69	2.18
49	7	-19.63	3.99	4.92**
56	5	-36.33	9.60	3.78*

*** $P < 0.001$; ** $P < 0.010$; * $P < 0.050$

Table 53 Comparisons of the migration indeces of leucocytes obtained from sheep infected with C. phagocytophila (Group 2) and sheep injected with normal saline (Group 1)

Days p.i.	Group One n	Mean	Standard deviation	Group Two n	Mean	Standard deviation	t
0	8	0.97	0.13	9	0.96	0.09	0.19
4	8	0.90	0.33	8	1.04	0.16	1.08
7	8	0.71	0.23	9	1.02	0.10	3.68**
10	8	6.75	0.19	7	0.94	0.07	2.49*
14	8	0.77	0.21	9	0.96	0.08	2.52*
17	8	0.83	0.21	7	0.95	0.06	1.45
21	8	0.79	0.28	7	1.02	0.08	2.09
24	8	0.58	0.25	7	1.01	0.04	4.48***
28	8	0.61	0.16	9	1.01	0.06	6.97***
35	8	0.70	0.26	9	0.99	0.05	3.28**
42	8	0.76	0.25	9	1.00	0.09	2.70*
49	8	0.76	0.20	9	1.04	0.07	3.96**
56	8	0.71	0.15	8	0.96	0.06	4.36***

*** $P < 0.001$; ** $P < 0.010$; * $P < 0.050$

p.i. = post injection (Group 1)

p.i. = post inoculation (Group 2)

Table 54 Significance of the differences of migration areas of leucocytes obtained before and after injection with normal saline and cultured without antigen (Day 0 - Day X)

Days post injection	Degrees of freedom	Mean difference (mm^2)	e	t
1	7	3.25	2.92	1.11
2	7	5.13	4.43	1.16
3	7	-2.50	4.84	0.52
4	7	-3.75	6.13	0.61

Table 55 Significance of the difference of migration areas of leucocytes obtained from sheep injected with normal saline (Group 3) and cultured with or without antigen

Days post injection	Mean difference (mm^2)	e	$t_{(7)}$
0	2.97	5.84	0.51
1	-2.63	7.62	0.35
2	2.00	5.04	0.40
3	1.50	4.84	0.31
4	1.38	6.28	0.22

Table 56 Significance of the differences of the migration areas of leucocytes obtained before and after inoculation with C. phagocytophila (Group 4; Day 0 - Day X)

Days post inoculation	Degrees of freedom	Mean difference (mm^2)	e	t
1	7	- 2.38	10.28	0.23
2	7	-20.75	11.15	1.86
3[a]	7	-60.13	7.99	7.53***
4	7	-62.25	5.70	10.92***

a = first day of parasitaemia *** $P < 0.001$

Table 57 Significance of the differences of migration areas of leucocytes of infected sheep (Group 4) cultured with or without antigen

Days post inoculation	Mean difference (mm^2)	e	$t_{(7)}$
0	-0.13	1.68	0.08
1	-5.25	6.58	0.80
2	-2.88	4.74	0.61
3	2.34	1.86	1.26
4	2.63	1.51	1.74

DISCUSSION

The migration of peripheral leucocytes of infected sheep was severely reduced during the period of parasitaemia. Evidence was obtained which suggested that the reductions in migration areas were directly related to the presence of sensitised lymphocytes. Because of the presence of cell-associated C. phagocytophila in some of the cells used for the migration tests, the addition of antigen to the cultures did not have dramatic effects and for this reason migration indices cannot serve as reliable indicators. Foster and Cameron (1970[b]), using the skin-window technique, found that the diapedetic ability of polymorphonuclear cells harbouring C. phagocytophila was reduced. They attributed this to the physical damage of the infected cells impairing the functional integrity of the cells. In the present study I found that the migration of the leucocytes of infected sheep was reduced by mediator substances released by antigen-sensitised lymphocytes. These substances reduced the migration of leucocytes of uninfected sheep.

The mechanism of inhibition of leucocytes have been investigated by many workers. Rocklin (1974), for example, demonstrated that the migration of human buffy coat cells was inhibited by soluble products of sensistised lymphocytes known as leucocyte migration inhibitory factors. These factors selectively inhibited human polymorphonuclear leucocytes and acted in a similar way to other lymphokines. Leucocyte migration inhibitory factors have recently been demonstrated in infections caused by E. canis (Nyindo et al., 1980). They found that the migration inhibitory

factors isolated from leucocyte cultures of dogs infected with E. canis had striking molecular and functional similarities to human and guinea pig leucocyte migration inhibitory factors. I did not attempt to purify the LMIF isolated in the present study but the specific inhibition of the migration of leucocytes from uninfected sheep left little doubt as to the true identity of the mediators.

The LMI test is characterised by variability and lack of reproducibility is a serious handicap of the test. In the present study I found great variations from day to day. The variations could have been due to technical changes from day to day such as speed of setting up the tests (Morley, Wolstencroft and Dumonde, 1974). Despite these variations however, with appropriate control measures reasonable conclusions can be drawn. That the reduction in migration areas of leucocytes obtained from infected animals irrespective of whether antigen was added or not were due to LMIF was confirmed by the fact that the supernatants of leucocyte cultures obtained during the peak period of parasitaemia (LMIF 1) specifically inhibited the migration of leucocytes of normal sheep.

The presence of cell-associated C. phagocytophila in the mononuclear cells made it impossible to find enough sheep with sterile immunity from which immunologically committed cells could be transferred. However, this finding, by itself, is important in so far as immunity and carrier state are concerned. Most of the animals which resist reinfection appear to be carriers (Foggie, 1951; Scott, 1978). In the present study four out of the five immune animals from which mononuclear cells were prepared and

injected into susceptible sheep were carriers. Measuring a carrier state is based on sub-inoculation of a few millilitres of blood. Foggie (1951), for instance, was sub-inoculating only one ml. Tuomi (1967) failed to detect carrier state in any of the cattle he tested when he was using one to six ml of blood but in one case where he used 500 ml he detected carrier state. It is conceivable that if small quantities of blood were used I would probably have missed some of the carrier sheep. Injecting large quantitites of mononuclear cells, seems, therefore, a better way of detecting carrier state when the concentrations of the organisms are very low.

No DTH reactions were observed in sheep infected with C. phagocytophila. Similar findings were reported by Nyindo and his colleagues (1980) in dogs infected by E. canis. These workers reported that the DTH response to 2, 4-dinitrochlorobenzene was reduced in dogs infected with E. canis. They speculated that this might be due to immunosuppressive effects of E. canis. The lack of DTH response in infections caused by C. phagocytophila merits further investigation. The failure of hypersensitivity reaction may be due to the nature and amount of antigen as well as the route of inoculations. Adelman, Hammond, Cohen and Dvorak (1979) suggested that polysaccharides do not induce delayed reactivity although they stimulate good antibody production and Leskowitz and Waksman (1960) reported that intravenous injections of antigen are less effective because they lead to tolerance to delayed hypersensitivity.

I did not investigate the correlation of leucocyte migration inhibition and humoral antibody response but the results suggested that CMI responses occurred significantly earlier than humoral response. Lewis and his colleagues (1978) suggested that humoral antibodies are effective only against cell-free ehrlichiae. The results in Chapter Seven showed that humoral antibodies play an important role against reinfection but the mechanism by which infections are eliminated, or at least brought to a state of tolerant symbiosis were not elucidated. The results in Chapter Two indicated that primary infections are characterised by the appearance of the organism in granulocytes and monocytes three to four days after inoculation and the parasitaemia stayed at peak levels for two to three days. The parasitaemia declined gradually in the next four to five days. The disappearance of infective organisms from peripheral blood coincided with the appearance of CF antibodies in abundance (Chapter Five) but the decline in the numbers of infected cells started much earlier. From these observations it is tempting to speculate that the clearance of the organisms starts early as a result of increased destruction of infective organisms and probably even infected leucocytes by lymphocyte-mediated cytotoxicity reaction as demonstrated by Kakoma, Carson, Ristic, Huxsoll, Stephenson and Nyindo (1977) in infections caused by E. canis. As antibodies are produced in abundance the destruction of the organisms is accelerated.

CHAPTER ELEVEN

GENERAL DISCUSSION AND CONCLUSION

INTRODUCTION

Studies on the immunity of animals against Cytoecetes phagocytophila have been hampered by the lack of reproducible serological methods (Tuomi, 167[d]). An investigation into ways of improving antigen yields and a comparative study of serological tests which have been used in other rickettsial infections were, therefore, considered necessary. Improved antigens yields were subsequently used to study the humoral and cell-mediated immune responses of sheep experimentally infected with C. phagocytophila and to differentiate ovine strains.

The in vitro studies originally designed to investigate the cultivation of the organism revealed important information about the morphology and stages of development of Cytoecetes phagocytophila.

IMMUNE RESPONSES

Humoral Antibodies

Our knowledge about the humoral immune responses of animals infected with C. phagocytophila is limited. Both the direct fluorescent antibody test (Tuomi, 1967[d]) and the complement fixation test (Snodgrass and Ramachandran, 1971) have been described but lack of reproducibility of the former (Snodgrass, 1974; Koske, 1976) and the unreliability of the antigen sources of the latter (Snodgrass, 1974) have hindered their wide application. In the present study I investigated ways of improving antigen yields and made a comparative study of four serological methods.

The induction of neutrophilia by treating infected sheep with the corticosteroid, betamethasone, resulted in an increase of the number of infected cells. Infected cells collected six hours after injection with betamethasone gave higher antigen titres. A further improvement in CF antigen titres was achieved by culturing infected blood at 37°C for 24 hours.

I made a comparative study of the CFT, the direct fluorescent antibody test, the indirect haemagglutination test and the enzyme-linked immunosorbent assay. Antigen-antibody reactions were detected by the first three methods but ELISA did not work because of the lack of purified soluble antigens. The CFT gave more reliable and reproducible results. The CF antibody titres were always higher than the IH antibody titres. The indirect fluorescent antibody test was complicated by excessive non-specific fluorescence but the direct fluorescent test was reproducible when the antibody titres of the sera from which the conjugate was prepared were high and the antigens were properly prepared. Because of its reproducibility the CFT was chosen as the test to use for further studies on the immune responses of sheep infected with C. phagocytophila.

Animals which had experienced an infection with C. phagocytophila develop an immune status of varying degrees. Some have a long lasting immunity (Stamp and Watt, 1950), while others are immune only for short periods (Hudson, 1950). The role of humoral antibodies in the protection against reinfection with C. phagocytophila has not been exhaustively studied hitherto. The only reported work on this aspect was that of Snodgrass (1974) who

suggested that the presence of CF antibodies was merely an indication of previous infection. His conclusions, however, do not have solid foundations. For example, he contended that sheep which were refractile to reinfection did not have detectable CF antibodies but it is not clear what he meant by resistance to reinfection. He took blood samples only when animals were showing a febrile response of above 40.5°C and by so doing he might have missed animals which had parasitaemic reactions without fever as I found in the present study. Furthermore, he claimed that 12 sheep which were challenged after ten months did not react, but the mean CF antibody titre after ten months was above 2^4, according to his tabulated data. In the present study I found that sheep whose reciprocal CF antibody titres were above 2^4 did not react with fever or with parasitaemia while those with reciprocal antibody titres of less than 2^4 reacted with a short period of parasitaemia which was sometimes accompanied with a short period of fever. The clinical reactions of secondary infections were found to be considerably milder than the primary reactions.

How humoral antibodies affect intracellular facultative organisms is controversial. Some workers believe that humoral antibodies coat naked organisms and thus reduce their ability to attach and penetrate host cells (Kreier, 1976). Humoral antibodies are also believed to enhance the phagocytosis of free organisms or even infected cells (Schroeder and Ristic, 1968). Some organisms which multiply extra-cellularly utilise surface characteristics to inhibit attachment to phagocytic cells; the polysaccharide capsule of pneumococci and the hyaluronic acid of

streptococci, for example, are believed to play such a role (Cohn and Hirsch, 1965). The importance of antibodies and complement in dealing with such organisms is well known. Other organisms readily get attached to phagocytic cells but avoid triggering the ingestion process. According to Jones (1974), for example, Mycoplasma pulmonis accomplishes this task through the help of a special protein in its cell wall. Other organisms avoid the phagocytic vacuoles by actively invading and lying inside the cytoplasm. Weiss (1973) speculated that members of the genus Rickettsia might bypass the action of the lysosomes by their active penetration into the cytoplasm and avoidance of the vacuoles. There are some organisms, however, that not only have the capacity to actively evade the defence mechanism based on the macrophages but have the ability to enter into the macrophages' lysosomes and create a micro-environment where they can survive and multiply. The organisms in the tribe Ehrlichiae possess this ability. Infections with Ehrlichiae are followed by the production of humoral antibodies but the effect of these antibodies on protective immunity is not clearly established. Du Plessis (1970), having failed to detect humoral antibodies in animals which had experienced infections with Cowdria ruminantium, claimed that the humoral antibodies did not play a role in the protection against reinfection. Snodgrass (1974) also claimed that the CF antibodies to TBF did not influence resistance to reinfection. However, contrary evidence exists. Organisms which invade and multiply inside vacuoles of macrophages are believed to have the ability to escape their destruction by partial or complete inhibition of the process of

fusion with macrophage lysosomes (Hirsch, 1972). Escape mechanisms of this nature have been demonstrated with the Chlamydia (Friis, 1972), Mycobacterium tuberculosis and Toxoplasma gondii (Jones, 1974). There is some evidence that C. phagocytophila possess the ability to inhibit lysosomal fusion. Tuomi and von Bonsdorff (1966) did not report changes in the structures of the granules of infected cells or note clear evidence of lysosomal fusion with the vacuoles containing the organisms. In the present study I found some degenerated organisms inside the vacuoles but there was no evidence of lysosomal fusion, indicating that lysosomal fusion is at least partially inhibited.

Some workers have presented experimental evidence that humoral antibodies affect the organisms' ability to inhibit lysosomal fusion. Gambril and Wisseman (1973) demonstrated that preincubation of Rickettsia mooseri with human typhus convalescent serum, which was not rickettsiocidal but which conferred passive protection to animals, opsonized the rickettsiae for enhanced phagocytosis by monocyte-derived human macrophages in cell culture and rendered them susceptible to destruction within the macrophages. Friis (1972) found that Chlamydia psittaci which were heat-inactivated or neutralised by antiserum were phagocytosed and quickly destroyed in contrast to those which were not treated; the latter multiplied vigorously inside the vacuoles. Lewis and his colleagues (1978) found that the addition of immune serum to macrophage cultures greatly reduced the multiplication of E. canis in macrophage cultures.

My findings indicated that the CF antibodies had a definite role in the protection against reinfection; protection was solid when the CF titres were high. The antibodies, however do not seem to affect some of the organisms within the cells. Blood collected from immune animals has been found to be infective to susceptible sheep (Foggie, 1951). In the present study I injected susceptible sheep with mononuclear cells from sheep which had resisted challenge reinfection, with the aim of passively transferring immuno-competent cells. The sheep reacted with fever and parasitaemia. In other words there exists a symbiotic relationship between the organism and the immune host to the mutual advantage of both; the organism survives inside certain cells without causing clinical damage to the host and the host develops resistance to reinfection. The moment the organisms are withdrawn from the immune environment and introduced to a non-immune environment, they regain their ability to invade new cells and provoke a clinical reaction which is followed by a "ceasefire and a negotiated settlement". The results in Chapter Ten seem to indicate that the inhibition of the organism inside the immune host from invading new cells was accomplished by the humoral antibodies. When up to 10^{10} "immune cells" among which were cells harbouring the organisms were inoculated into non-immune sheep they failed to give protection.

A definitive examination of the role of humoral antibodies in the protection against reinfection requires a passive transfer of antibodies to susceptible animals or some type of neutralisation test <u>in vitro</u>. I did not attempt to transfer antibodies passively

because the number of experimental animals required was prohibitive and because of the lack of standardised cell-free organisms. The *in vitro* neutralisation test requires cell-free organisms upon which antibodies may act and susceptible culture systems or experimental animals. Such tests will be feasible only when cell culture systems and cell-free organisms are available.

Cell Mediated Immunity

Since Mackaness (1969) elegantly demonstrated the importance of cell-mediated immunity for the killing of intracellular facultative parasites the importance of this type of immunity has been widely recognized. This type of immunity can be transferred to non-immune recipients by injecting lymphocytes from an immune animal. It is believed to be mediated by T-cells (Howard, Mattsson, Seidel and Balfour, 1978). Using the leucocyte migration inhibition test Buening (1973) and Carson and his colleagues (1977) demonstrated CMI response in cattle infected with *Anaplasma marginale*. Nyindo and his colleagues (1980) postulated that humoral antibodies are effective only against cell-free ehrlichiae; destruction of intracellular organisms being related to events within the infected cells. However, they failed to find a direct correlation between the presence of increased humoral antibody titres and cell-mediated immune responses, as measured by the leucocyte migration inhibition test. Kakoma, Carson and Ristic (1980), in a review of the participation of lymphocytes in the immunity and immunopathology of *E. canis*, speculated that specific and non-specific migration inhibition did not prevent clinical

disease but might be related to protective immunity; recovered dogs maintained high CMI responsiveness but continued to harbour the organisms in the spleen, lymph nodes and liver and this ensured a continuous antigenic stimulation of the effector immunocytes.

In the present study a CMI response to C. phagocytophila as measured by the LMI test was demonstrated during the clinical manifestation of the disease and remained at high levels for a long time after the cessation of clinical signs. The CMI response was detected earlier than the humoral response. The relationship of CMI and protective immunity was not specially studied, but two sheep which were challenged four months after infection when the CMI response was strong did not react clinically. The humoral antibody titres at the time of challenge were also high.

Carrier State and Protective Immunity

That the blood of animals which have experienced a natural or experimental infection with TBF remains infective for some time is well established. However, our knowledge on the duration of the carrier state and its possible immunological importance is fragmentary. Nevertheless, the carrier state has an important role on the epidemiology of the disease; recovered animals which are refractile to reinfection can serve as reservoirs.

It has been speculated that for animals to develop lasting immunity they must be continuously exposed to the disease (Gordon et al., 1932). Many workers accepted that while the phase of premunition lasted the animal resisted reinfection (Foggie, 1951) but

recent evidence indicates that immunity to TBF is either as a result of a carrier state or due to a transient sterile immunity (Scott, 1978). The immunity to TBF appears to be similar to that described by Neitz and his colleagues (1947) for heartwater. According to these workers heartwater has two phases of immunity: a short but variable period of premunition or carrier state followed by a phase of gradually decreasing sterile immunity. They also showed that during the period of declining sterile immunity infection resulted in the reappearance of the infective agent in the circulating blood and this initiated a repetition of the cycle of premunition and sterile immunity. Most reports on the carrier state are based on isolated observations on few animals. The few attempts to investigate the problem experimentally did not give clear information because most were not well designed. Foggie (1951), for example, after observing a field case where the blood of one sheep was infective for 25 months, set out to investigate the problem. He inoculated ten sheep with infected blood and then bled them every week. He pooled the blood and inoculated one susceptible sheep with one ml of the pooled blood. The pooled blood was infective for 27 weeks, and occasionally for periods as long as 38 weeks. However, because he pooled the blood it is not possible to know how many of the animals became carriers and for how long. Another problem in ascertaining the carrier state has been the amount of blood required for sub-inoculation. Tuomi (1967[a]), for example, reported that while he could not isolate the organism from cattle which had experienced infection by sub-inoculating three to six ml of blood, he isolated

the organism from one calf by sub-inoculating 500 ml of blood but he did not pursue the problem further. Foggie (1951) demonstrated carrier state by splenectomy and Scott (1978) used immunosuppressive drugs to the same end.

Scott (1978) found that sheep infected with C. phagocytophila became carriers after recovery from the clinical disease. The carrier state persisted for several weeks in all sheep. Sheep challenged during the period of premunition resisted reinfection but he also claimed that 12 months after primary infection 30 percent of those animals which resisted reinfection were not carriers.

In the present study when sheep were challenged when their reciprocal CF antibody titres were below 2^4, some reacted with fever and parasitaemia, some reacted with a mild parasitaemia while others did not react at all. The carrier status of these animals was not tested before challenge but it is likely that those animals which reacted with parasitaemia were sterile before challenge while those which did not react were either sterile or carriers.

E. canis is believed to cause a carrier state of a long duration (Ewing and Buckner, 1965). Buhles and his colleagues (1974) reported that dogs inoculated with blood of dogs infected as long as five years earlier developed the disease. E. equi, on the other hand, has not been reported to cause a carrier state even of a short duration, despite the alleged long periods of immunity against reinfection (Gribble, 1969; Lewis, 1976).

C. phagocytophila appears to behave like E. canis in causing carrier state and like E. equi in causing sterile immunity. It is interesting to note that E. equi exclusively parasitises the granulocytes (Lewis, 1976) while E. canis invades the mononuclear cells. In contrast, C. phagocytophila, while predominantly infecting the granulocytes also affects the monocytes. It is conceivable that unless other sites of multiplication during the period of a carrier state exist, the proliferation of the organism for long periods is possible only when the monocytes are invaded. After all the mature granulocytes are end cells whose half life after arriving in the peripheral blood is not more than seven hours (Hirsch, 1972). The mononuclear cells have a longer life in the tissues and under certain conditions they even divide (Hirsch, 1972; Chapter Three). In the present study, I found that the monocytes were invaded at the later stages of the disease. Most sheep had few monocytes infected but in few sheep no infected monocytes were detected. There is some evidence which suggests that a minimum period of proliferation of the organism is needed for the development of immunity. Synge (1976) found that if a drug was administered which eliminated the infection early in the course of the reaction no protective immunity developed. Alternatively if a drug was used which only suppressed the organism a relapse reaction occurred. Similar observations were reported by Amyx, Huxsoll, Zeiler and Hilderbrandt (1971), in infections with E. canis. They treated 15 dogs with tetracycline immediately after they had developed parasitaemia. All but two of them were freed from infection but when they were challenged later they reacted

as if they did not experience the disease. Laeflang (1972) treated three dogs with oxytetracycline after they had recovered from a clinical infection with E. canis, but while they were still carriers. The drug eliminated the carrier state but when the animals were challenged they regained their carrier status without showing clinical reactions. Neitz (1968) claimed that drugs did not interfere with the development of immunity against infections of Cowdria ruminatium but Synge (1976) postulated that when animals infected with C. ruminatium were treated with a drug which eliminated the organism the development of immunity might be impaired as in TBF. Similarly Castillo and Martinez (1972) reported that treatment of bovine anaplasmosis was satisfactory when the carrier state was not completely eliminated because immunity developed.

In the present study I found that the CF antibodies in the IgM class persisted for periods exceeding 12 months. This is most likely due to the continued presence of C. phagocytophila in the immune animals. Following infections with viruses and bacteria IgM antibodies are produced before IgG antibodies but they do not persist unless there is a continued stimulation by the antigen (Murphy et al., 1966; Uhr and Finkelstein, 1967).

Testing for the carrier state has been difficult to standardize because of the need to inoculate large quantitites of blood or the need to splenectomize or to use immunosuppressive drugs. It is also possible that the organism proliferates in sites other than blood, appearing in the bloodstream only periodically. In the latter case inoculation of blood might not be the appropriate

test for the detection of carrier state. Detection of CF antibodies on the IgM class of immunoglobulins months after infection, on the other hand, is easy to perform and accurate. Measurement of IgM antibodies specific for defined antigens are being increasingly used as a diagnostic tool for differentiating recent infections from past experience of infections (Dittman, Cleary and Castro, 1979).

Detection of CF activity on the IgM class months after the cessation of clinical reactions, might, therefore, serve as a measure of the carrier state.

STRAIN DIFFERENTIATION

Many workers had suspected that different strains of C. phagocytophila existed, especially after the recognition of the disease in cattle (Hudson, 1950). However, differentiation between the suspected strains has proved difficult. The main problem was lack of clearly defined parameters for assessing differences. The problem was further aggravated by the poorly designed experiments, many workers embarking on the differentiation of strains without having established base-line data for at least one of the strains. Assessment of differences was usually based on cross-immunity trials, animals which had experienced infection with one strain being challenged by another. Since the duration of immunity to homologous challenge was not firmly established, the reports of cross-immunity were often conflicting. While Tuomi (1967[c]), for example, was claiming that no strain gave cross-protection to another strain, Foster and Cameron (1970[a]) found two strains

immunologically identical.

Foggie (1951) reported that five sheep previously infected with the bovine strain reacted to challenge inoculation by an ovine strain, compared to four sheep which were infected with an ovine strain and did not react to homologous challenge. Foggie and Allison (1960) reported that there was no evidence of protection against ovine strains following infections with bovine strains but their allegation was based on two sheep infected with a bovine strain which reacted to challenge by an ovine strain after an unspecified period. Tuomi (1967[b]) reported that Finnish bovine strains did not confer protective immunity against Scottish ovine strains.

Some workers have attempted to differentiate strains on the basis of their virulence, but measuring virulence on such a benign disease as TBF has proven difficult. Foggie (1951), for example, said that depending on the virulence of the infecting strain, the temperature might drop rapidly or might show a more gradual fluctuating decline lasting ten to twelve days. He also speculated that the strains of TBF might vary greatly in virulence and that pneumonia tended to appear in epidemic form in sheep which had experienced attacks of the disease. Tuomi (1967[b]) and Foster and Cameron (1970[a]) tried to measure differences in virulence among bovine and ovine strains by the length of the incubation period, the degree and duration of fever, the proportion of infected leucocytes and the duration of parasitaemia, and the degree of neutropaenia. Tuomi (1967) claimed that he found differences but Foster and Cameron (1970[a]) did not.

Nevertheless, there is little doubt that different strains exist. Strain differences are a common feature of many rickettsial organisms. Strains of many rickettsial organisms have been antigenically differentiated. The complement fixation test, for example, is widely used to differentiate and characterise group and type specific antigens of chlamydias (Dhir, Kenny and Grayston, 1971) and rickettsias (Dasch and Weiss, 1977). After purifying rickettsias many workers (Kobayashi et al., 1969) have managed to separate their antigens to group and type specific, and thus to differentiate and type strains. Type differentiation, using the complement fixation test as a tool, has also found wide use in the typing and subtyping viruses (Davie, 1964).

The scope for antigenic differentiation of strains of C. phagocytophila has been, hitherto, limited by the lack of reproducible serological tests. Tuomi (1967[d]) reported that using the direct fluorescent antibody test, he could not detect antigenic differences between various strains but he speculated that if more refined techniques were applied, including dilution and adsorption with heterologous strains of the conjugate, and the use of the indirect method, antigenic differences between immunologically deviating strains could be discovered. Snodgrass (1974) thought that if antigenic similarities could be demonstrated between strains of C. phagocytophila by the CFT, the test would have an immense diagnostic value but he did not undertake any study to investigate antigenic relationship or differences among strains. In the present study the improvement in antigen yields made it possible to undertake such a study. Using the CFT I found very strong

antigenic relationships among three ovine strains of C. phagocytophila. Despite the strong antigenic relationships however, quantifiable differences were obtained by comparing the antibody titres of sera against homologous and heterologous antigens.

I attempted to break down the antigens to soluble and particulate forms with acetone-ether extraction according to the methods used to characterise chlamydial and rickettsial antigens (Kobayashi et al., 1969) without success. I believe that this can only be accomplished when the organisms are available in their purified forms without contamination with host cell contents.

The fact that different strains have similar antigenic components has a significant diagnostic value (Snodgrass, 1974). According to Tuomi (1967[c]) the heterogeneity of strains of C. phagocytophila might have no parallell with any other species of micro-organisms. Under these circumstances, differentiation of strains is of utmost importance for the epidemiological surveillance of the disease and for any future attempts to prevent outbreaks of the disease by vaccination. I believe the method contributes a new approach of resolving the perennial and controversial problem of antigenic differentiation.

IN VITRO CULTIVATION

Attempts hitherto to propagate C. phagocytophila in vitro have been unsuccessful (Thrusfield et al., 1978). In the present study no evidence was obtained as to whether new cells were invaded in vitro. The whole blood cultures, however, provided ample evidence that the organism continued to multiply in

granulocytes and monocytes *in vitro*. These whole blood cultures have proved very useful in the morphological characterisation of the organism and the improvement of antigen yields. Transient culture systems have proved useful in improving antigen yields from other facultative intra-cellular parasites. Despite the smallness of the increase in the number of infected cells, Hall, Haynes, Chulay and Diggs (1978), for example, used whole blood cultures to improve yields of antigen for *Plasmodium falciparum*.

C. phagocytophila, like other related organisms such as *E. equi* and *E. canis* is characterised by a high degree of target cell specificity. What is more intriguing about these organisms is their choice of granulocytes and monocytes as their target cells. While *E. equi* specifically parasitises the granulocyte (Lewis, 1976), *E. canis* prefers the lymphocytes and monocytes but has also been reported to have been found in the granulocytes (Ewing, Robertson, Buckner and Hayat, 1971). *C. phagocytophila*, while showing strong morphological similarities to *E. equi*, has its target cells primarily the granulocytes but it is also found in the monocytes. Ristic (1976) suggested that the ability of the host cell to replicate is an essential requirement for the development of culture systems for these organisms. He sited the success of the propagation of *E. canis* in monocytic cultures (Nyindo *et al.*, 1971) as an example. Weiss (1973), on the other hand, thought that although rickettsiae require well nourished host cells, the host cells need not multiply.

For a successful penetration of host cells some rickettsiae require more than casual contact with the host cell during the

early phases of infection. For example, contact with cells was accomplished by centrifuging rickettsiae on to the cells at 1500 g for one hour (Weiss, 1973). However, while active participation of some rickettsiae is required, the entry of others is accomplished entirely by the host cells. Friis (1972), for example, concluded that the entry of Chlamydia psittaci into murine fibroblasts was brought about entirely by the phagocytic activity of the host cells. It has been speculated that active penetration of the host cells is unlikely in infections by C. phagocytophila in vivo (Tuomi and von Bonsdorff, 1966). According to Moulder (1974) the intracellular organisms having acquired fitness traits enabling them to survive inside cells have lost the ability to survive outside them. However, their objective of moving from one host to another is accomplished either by acquiring an arthropod vector to transport them from one host to another safely or as in the case of Coxiella burnetii and the Chlamydiae by having a small hardy particle which survives outside and a cell-adapted one which multiplies inside the host cell.

The failure to propagate C. phagocytophila in the fibroblast-like cell lines derived from peripheral blood monocytes might have been due to the loss of the phagocytic activity of such cells. To allow a better contact between the fibroblast-like cells and the organism I co-cultured infected cells and cell lines derived from peripheral blood. Apart from an intense eosinophilic granulation no definite evidence of multiplication of the organism in the fibroblast-like cells was obtained. Moreover, granulation was also observed when normal leucocytes were co-cultured with

the fibroblast-like cells. Infected granulocytes and monocytes were observed lying side by side with the fibroblast-like cells without the organism being established in the fibroblast-like cells for up to 48 hours. Similar attempts to co-culture infected leucocytes with spleen or lung cells did not produce viable infections.

No information is available about the viability of cell-free forms of C. phagocytophila. Foggie (1951) reported that plasma and serum filtered through 1.5 microns were infective. I undertook preliminary studies to investigate the viability of cell-free forms of the organism. I filtered plasma and serum of infected sheep through 1.2 microns and divided them into three equal samples. One sample was immediately inoculated into susceptible sheep, the second was left at 37°C for two hours and then inoculated and the third was left at 4°C for 24 hours before being inoculated into sheep. Sheep inoculated with the first two samples reacted with TBF while the sheep which was inoculated with the sample left at 4°C for 24 hours did not react.

The results in this study suggested that the organism continues to multiply once inside granulocytes and monocytes in vitro and may possibly multiply in newly infected active phagocytes. This hypothesis was reinforced by my finding that particles and clusters with morphological characteristics of C. phagocytophila were present in monocytic cultures from infected sheep for up to 14 days after culture. These particles were passaged four times onto monocytic cultures derived from normal uninfected sheep. Similarly Scott and Munro (1975) had observed that leucocytic

cultures from infected sheep showed granules for a few days but the cultures were not infective to sheep. These observations merit further investigation. Future attempts to continuously propagate the organism should concentrate on phagocytic cells.

PATHOGENESIS

Our knowledge of the pathogenesis of TBF is fragmentary. Remarkably few studies on the pathology of the disease have been reported other than the haematological changes which accompany the disease (Taylor et al., 1941).

Although the organism parasitises predominantly the neutrophils it has also been reported to affect the eosinophils and monocytes. However, the degree of parasitism of the eosinophils and monocytes has not been clearly defined. Purnell and Brocklesby (1978) described a virulent strain of TBF characterised by what they claimed was a marked invasion of monocytes of splenectomised calves. However, their observations of maximal infection rates of 46 percent of the neutrophils and 27 percent of the monocytes are not higher than the rates commonly observed with ovine strains of TBF. The results from my study suggested that the monocytes were invaded at the later stages of parasitaemia. This period also coincided with the period of monocytosis but infections of the monocytes at early phase of the infection were not uncommon. The haematological changes and the sequential infection of the cells suggest that either the organism prefers one series of cells or the defence mechanism is such that the granulocytes are in the first line of defence and, therefore, get infected prior to the

monocytes. Detection of infected eosinophils is relatively difficult because of the masking by the granules. Nevertheless, I noticed that the eosinophils were infected relatively early. Moreover, the eosinophils disappeared from the circulation at the onset of parasitaemia. Tyzzer (1938) observed that the eosinophils furnished conditions more favourable to Cytoecetes microti than the neutrophils. From these observations it is conceivable that the eosinophils are affected first, with the neutrophils and monocytes, in that order, being affected subsequently.

The mechanism by which the disease causes leucopaenia is not clearly established. Taylor and his colleagues (1941) speculated that it was caused by marrow aplasia and not by exhaustion because they found that neutrophilia was induced by the injection of bacterial toxins but they could not explain the acute lymphocytopaenia. While Taylor and his colleagues (1941) claimed that there was no shift to the left, Hudson (1950) reported that neutropaenia was accompanied by the increase in the number of cells with unlobed nuclei and ribbon-nucleated cells. Some workers have implied that neutropaenia is due to the destruction and sequestration of infected cells. Hudson (1950) observed a tendency to swelling and frying of the nuclei, suggesting that the granular leucocytes were undergoing pathological changes but he added that this might not be due to the actual presence of the organism in the cells. Foster and Cameron (1970[b]) presented experimental evidence which suggested that the functional integrity of neutrophils might be affected by the presence of the

organism. Tuomi and von Bonsdorff (1966), on the other hand, did not find any structural damage except vacuolation which could be attributed to the presence of the organism.

In the present study, evidence was obtained that there was no hypoplasia of the bone marrow, at least during the early phases of infection. When infected sheep were injected with the corticosteroid, betamethasone, neutrophilia was induced. I did not find any structural damage other than vacuolation that could have been caused by infection with C. phagocytophila. Buhles and his colleagues (1974) suggested that the pancytopaenia of the acute phase of canine ehrlichiosis might be analogous to that caused by acute cases of viral and bacterial infections which result in the increased sequestration or destruction of cells rather than decreased production, while that of chronic canine ehrlichiosis might be due to bone marrow hypoplasia (Hilderbrandt et al., 1973). Chronic cases of pancytopaenia have not been reported in TBF; the disease is acute and the haematological changes very sharp. Although the explanation based on the destruction of the granulocytes as the result of infection is plausible, the lymphocytopaenia requires further investigation. It is possible that the organisms might have endotoxins. Weiss (1973) reported that rickettsiae have endotoxic activity like other gram-negative bacteria. The presence of endotoxin might partially explain the lymphocytopaenia and the febrile reaction. However, the febrile response may be directly attributable to the accelerated destruction of infected granulocytes. Many workers have presented evidence which suggested that the principal factor

in diseases that raises the set point of temperature was an endogenous pyrogen derived from the leucocytes (Wood, 1970; Aitkins and Bodel, 1972). In the present study I found that the febrile reaction was dramatically reduced, albeit temporary, when a corticosteroid was injected.

An unanswered question is where the organism proliferates before the peripheral blood becomes infective. Snodgrass (1974) found that the lung tissue was infective before blood and the bone marrow. In the studies designed to investigate improvement of antigen yields, I found that the granulocytes which entered the circulation from the bone marrow reserve were not infected before arrival at the circulation. It seems that the leucocytes are infected in the circulation probably as a result of phagocytosis but the role of the other sites of proliferation merits further investigation. Ewing (1969) suggested that autoradiographic techniques might be useful in the determination of primary sites of multiplication of E. canis but no one has followed up his suggestion. I made sequential histological studies using the direct immunofluorescent technique of sheep infected with C. phagocytophila. Preliminary results showed that the lymph nodes were possibly the primary targets but because of the limited numbers of animals used no definitive conclusions were drawn.

The organism persists in the peripheral blood for a long period after recovery (Foggie, 1951) but whether the organism continues to proliferate in other sites is not known. Other related organisms have been reported to proliferate in the reticulo-endothelial macrophages. Mugera and Kiptoon (1978)

found C. ondiri in the capillaries of the endothelial cells of the liver, spleen and kidney and in the macrophages of the reticulo-endothelial systems. Ehrlichia canis was found in more abundant numbers in the lung macrophages than in the blood (Ewing, 1969) and Du Plessis (1976) reportedly found Cowdria ruminantium in the lymph node cells of sheep and the peritoneal macrophages of mice. Several workers have postulated that the persistence of the intra-cellular facultative organisms inside well-preserved macrophages induced a prolonged premune state and the likely site of this long term latency was the reticulo-endothelial system. Kordova (1978) argued, for example, that Chlamydiae and Rickettsiae were taken up by macrophages and then managed to survive inside the cells by changing into cell-wall defective variants. But how do these organisms manage to stay in a hostile environment, apparently unable to proliferate by invading new cells and yet able to initiate new infections when inoculated into a new host? The question of latency is an intriguing one. Many workers have attempted to answer. Thus, Kordova (1978), in a review of Chlamydiae and Rickettsiae, raised an interesting theory which attempted to answer why obligate intra-cellular microbes induced latency and why or how inapparent infections converted into active disease. She argued that Chlamydiae and Rickettsiae were gram-negative bacteria which had cell-wall defective variants particularly adapted to intra-cellular life. Cell-wall defective variants of many bacteria are commonly produced, for example, by treatment with penicillin. When the cell-wall defective variants are adapted to parasite life they have an added advantage in that they might

be able to escape the defence mechanism of the host which is largely directed towards the cell-wall components. Moulder (1969) argued that the elementary bodies of the Chlamydia have rigid cell walls analogous to ordinary gram-negative bacteria but the large non-infectious forms did not have rigid cell walls and that the large ones can be regarded as naturally occurring L-forms of bacteria. That the large forms can be L-forms was reinforced by Tanami and Yamada's (1973) findings. When they added penicillin to chlamydial cultures, it failed to arrest the reproduction of chlamydial large forms but transformed the small into large forms which reproduced by budding and/or by a process similar to endosporulation. Kordova (1978) thought that under certain conditions Chlamydiae converted into cell-wall defective variants and that the primitive discriminatory capacity of the macrophages (Mackaness, 1976) did not allow them to distinguish the organisms with deficient cell wall from "self". She concluded by saying, "we think that cell wall defective variants of Chlamydiae and Rickettsiae, including the minute, infectious unit of Coxiellae, are responsible for inapparent or latent infections of healthy carriers whereas differentiation into 'bacterial' forms causes manifest disease under appropriate conditions".

If Kordova's (1978) theory holds, then one can extend it to the carrier state of TBF and other macrophage-associated organisms. If we assume that the small forms are the infective ones and the large are cell-wall defective variants the following immunological scenario becomes plausible: when susceptible animals are inoculated with C. phagocytophila for the first time, the infective small

forms are phagocytosed. They are then quickly transformed to the large cell-wall defective forms within the vacuoles and they multiply by binary fission. After a period some of these large forms regain their cell-wall rigidity and leave the vacuole to initiate new infections. In the meantime the host develops immune mechanisms, humoral antibodies, killer cells, etc., particularly directed towards the small forms and as immunity develops further invasion by the small forms becomes reduced. Some of the organisms in the macrophages continue to multiply at a reduced rate to give small forms which are unable to invade new cells because of the presence of antibodies. However, when macrophages harbouring the cell-wall defective large forms are withdrawn from the immune environment and introduced into a new, non-immune host they multiply by binary fission; some are transformed into small infective forms which in the absence of antibodies are able to invade new cells.

DEVELOPMENT AND CLASSIFICATION

The pleomorphism of C. phagocytophila is well known and documented (McEwen, 1947; Hudson, 1950; Foggie, 1951; Gordon et al., 1962). This led many workers to postulate the existence of an elaborate developmental cycle. Gordon and his colleagues (1962), for example, thought there was a stadial progression from 'initial bodies' to 'morulae' and then to infective 'elementary bodies'. The nomenclature for the different morphological types was borrowed from those used by Bedson and Bland (1932) to characterise what they considered were developmental stages of

groups of organisms in the genus Chlamydia. Other organisms which closely resemble the agent of TBF have been similarly characterised. Donatien and Lestoquard (1935) first used this nomenclature for E. canis and this has been retained by others.

Tuomi and von Bonsdorff (1966) threw more light on the morphology of the organism. They found the organisms in cytoplasmic vacuoles. These vacuoles were clearly delineated from the host cytoplasm and contained single or multiples of particles, indicating that what were appearing as 'initial bodies', 'morulae' etc. were no more than particles of various sizes, either tightly packed together or loosely arranged inside a big vacuole. Despite this, however, they maintained there was a life cycle, albeit different from that of the Chlamydia. No other ultrastructural study on the organism has been reported in the literature but more information is available from other organisms closely related to TBF. Kraus and his colleagues (1972) reported that Cytoecetes ondiri, the causative agent of bovine petechial fever, was found in cytoplasmic vacuoles of polymorphonuclear cells and that it divided by binary fission and by condensation of small dense bodies from big, giant bodies. They claimed that there were some similarities to the life cycle of the Chlamydiaceae but that the elementary bodies of C. ondiri were bigger in size.

Ultrastructural studies have also been carried out on Ehrlichia canis (Hilderbrandt, Conroy, McKee, Nyindo and Huxsoll, 1973) and on Ehrlichia equi (Sells et al., 1976). These organisms are very similar to C. phagocytophila and according to the latest edition of Bergey's Manual of Determinative Bacteriology (Philip, 1974)

they are all included in the genus Ehrlichia; the causative agent of bovine petechial fever was not included. Hilderbrandt and his colleagues (1973) called the particles 'elementary bodies' but they did not establish the existence of 'initial bodies' or 'intermediate bodies' as described for the Chlamydia. They claimed that the ultrastructure of E. canis was similar to the other members of the genus Rickettsia and large particles of the genus Chlamydia. Sells and his colleagues (1976) claimed that the ultrastructure of E. equi was similar to that of C. phagocytophila, C. ondiri, E. canis, to the organisms in the genus Rickettsia and to the large particles of the genus Chlamydia but they could not establish a life cycle.

Although the ultrastructural studies have given invaluable information they have so far failed to resolve the question of developmental cycle and proper classification. I approached this problem in two ways. My first attempt was to investigate whether the morphological types seen under the light microscope appear in a sequential manner. The ability of the organism to continue to propagate in vitro for at least 24 hours, gave a hitherto impossible advantage of isolating a certain sample and studying the morphological changes that occur in the infected cell outside the animal. By studying the morphological types before and after culture it became clear that there was, indeed, a sequence of development from single discrete particles to clusters of particles. My second approach was to ultrastructurally characterise the morphological types seen under the light microscope. The ultrastructural findings showed that the organisms were found inside

clearly defined vacuoles as single particles or clusters of particles. The number, size and arrangement of these particles inside the vacuoles varied, thus explaining the pleomorphism of the inclusions under the light microscope. The particles divided by binary fission and small dense particles were found outside the vacuoles suggesting that they are the likely ones which leave to infect new cells. The findings in this study suggested that the developmental cycle of C. phagocytophila is simple. The organisms are phagocytosed by granulocytes and monocytes and are then enclosed in the invaginating membrane which develops into a vacuole. The particles enlarge and divide by a binary fission and they grow in numbers, giving rise to what appear as 'morulae' or 'clusters'. Some particles leave the vacuole to initiate new infections.

The existence of small dense bodies which are similar to those bodies popularly known as elementary bodies (EB) in the Chlamydia, raises the question which has been speculated for many years. Do they represent a necessary stage of development without whose presence new cells would not be invaded? Becker (1978) compared the EB of the Chlamydia with the spores of some bacteria, their role being to withstand extra-cellular conditions, multiplying only when favourable conditions prevail. Kordova (1978) theorized that the large intra-cellular forms were not different from other cell-wall defective bacteria which divided by binary fission and could be infective to new cells only when they have transformed into the EB which did not divide but were capable of withstanding extra-cellular conditions and initiating new

infections. In the present study despite the observation of numerous small particles none were found in the process of division while big particles were found on the process of binary fission and suspected cases of budding and condensation. How the process of change from big particles to small particles takes place requires further investigation. Kraus and his colleagues (1972) described a process of condensation of small dense bodies from large particles but I did not find definitive cases of condensation.

Classifying the organism solely on the basis of its location inside cytoplasmic vacuoles and the presence of two types of particles would suggest that it is closer to the Chlamydia than to the Rickettsia because the latter are large elongated particles which, except for R. sennetsu, are not enclosed inside vacuoles (Anderson et al., 1965). However, the organism differs from the Chlamydia in other ways. The Chlamydia are rarely transmitted by arthropods while TBF is transmitted by ticks and the Chlamydia readily grow in embryonated eggs and cell culture systems while C. phagocytophila has not been propagated in ovo and it has only been transiently propagated in its natural target cells. No antigenic relationship has been demonstrated between the Chlamydia and TBF (Foggie, 1962[b]). Using the CFT I found that C. phagocytophila antigens did not react with high titred immune sera raised against the chlamydial agent of enzootic abortion of ewes. C. phagocytophila also has other properties which separate it from the organisms of the genus Rickettsia. Ectoparasites transmit rickettsia to man and some rodents through their faecal

contamination while C. phagocytophila and other related organisms are transmitted to animals by ticks. Because of these differences the 'rickettsia-like' organisms of animals have been separately included in the tribe Ehrlichiae (Philip, 1974). Some of these 'rickettsia-like' organisms have stronger similarities to TBF than others. For example, the organism described in the granulocytes of voles by Tyzzer (1938) has striking similarities to C. phagocytophila. Both organisms are found in the granulocytes and they are morphologically similar, if not identical, (Foggie, 1962[a]). Kraus and his colleagues (1972) proposed that the causative agent of bovine petechial fever be classified as Cytoecetes ondiri on the basis of its morphological similarities to C. microti (Tyzzer, 1938). Their argument was further supported by Mugera and Kiptoon (1978). Snodgrass (1974), after a comparative study of TBF and bovine petechial fever, concluded that the causative agent of petechial fever was another strain of C. phagocytophila. Gribble (1969) described another organism with striking similarities to TBF in horses in California. This organism which has been classified as Ehrlichia equi appears more related to C. phagocytophila than E. canis. Unlike E. canis which affects only the Canidae, it has been transmitted to sheep and goats (Gribble, 1969) and to dogs, cats and non-human primates (Lewis, Huxsoll, Ristic and Johnson, 1975) but what is more interesting is that while E. canis invades mononuclear cells E. equi has been demonstrated only in the granulocytes.

Ewing and his colleagues (1971) described a strain of E. canis which was allegedly found principally in circulating neutrophils

and eosinophils rather than in lymphocytes and monocytes. The agent described by the last authors is reported to be morphologically indistinguishable from C. phagocytophila and E. equi (Lewis, 1976). Until such time when the organism is available in its pure form for biophysical and biochemical characterisation and final classification, I propose that it should be classified in the genus Cytoecetes created by Tyzzer (1938) along with the causative agent of bovine petechial fever and equine ehrlichiosis to differentiate them from the other members of the tribe Ehrlichiae which invade the mononuclear cells.

CONCLUSION

Lack of reliable sources of antigen has been recognised as a major obstacle to immunological studies of tick-borne fever (Tuomi, 1967; Snodgrass, 1974). I found that a combination of treating infected sheep with the corticosteroid, betamethasone and culturing infected blood at 37°C for 24 hours improved antigen yields significantly. In the light of improved sources of antigen a study of the immune responses of sheep infected with C. phagocytophila became feasible.

Four serological tests were compared. Antibodies were detected by the direct fluorescent antibody test, the complement fixation test and the indirect haemagglutination test. The last test has not been previously described. The complement fixation test was found to be more sensitive and reproducible while the direct fluorescent antibody test was found useful for in vitro studies.

The CFT was used to study the duration of humoral immune response. Antibodies were first detected two weeks after infection and persisted for as long as 18 months after infection. The relationship of CF antibodies and protective immunity was studied by challenging groups of animals after their CF antibody levels had reached previously specified levels. The threshold of immunity was found to be around a reciprocal CF antibody titre of 2^4. The kinetics of antibody production of sheep experimentally infected with C. phagocytophila was studied by fractionating sequential sera into IgM and IgG and testing them for CF activity. It was

found that initial antibody response was mainly on the IgM fraction but was quickly followed by IgG; the IgG becoming dominant after six weeks post-inoculation. A significant finding was that CF activity on the IgM fraction persisted for as long as 12 months. The last finding was attributed to the carrier state.

The complement fixation test was also used to differentiate strains of C. phagocytophila. All three strains tested cross-reacted suggesting that they shared common antigens but quantifiable differences were established by titrating immune sera against homologous and heterologous antigens. I believe that the last finding will be useful for the epidemiological surveillance of outbreaks of TBF and for any future attempts of preventing disease outbreaks by vaccination.

A hitherto undescribed in vitro test for CMI response of sheep infected with C. phagocytophila was developed. Sheep infected with C. phagocytophila showed CMI response, as measured by the leucocyte migration inhibition test, significantly earlier than humoral immune response, as measured by CFT.

Finally, I venture to speculate that immunity to C. phagocytophila is a result of collaboration between cell-mediated and humoral immunity. The early reduction of parasitaemia is likely due to CMI but the clearance of the organism and the subsidence of clinical reaction do not occur until ample antibodies are produced.

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Appendix I

Preparation of Solutions

Alsever's Solution

D-glucose (dextrose)	2.050 g
Sodium citrate	0.800 g
Sodium chloride	0.420 g
Citric acid	0.055 g
Distilled water	100 ml

Autoclave at 15 lbs per square inch for 15 minutes and store at $4^{o}C$

Richardson's Reagent

Stock solution A:- Borax ($Na_2 B_4 H_7.10H_2O$).....0.57 g

Sodium azide (NaN_3) .....0.81 g

Dissolve in saturated NaCl and make up to 100 ml

Stock solution B:- Boric acid ($H_3 BO_3$) .....0.93 g

Borax ($Na_2 B_4 H_7.10H_2O$) .....2.29 g

Sorbitol ($C_6 H_{14} O_6.\frac{1}{2}H_2O$) ...11.74 g

Dissolve in saturated NaCl solution and make up to 100 ml

To preserve complement mix eight parts of guinea pig serum with one part of solution A and then add one part of solution B and store at $4^{o}C$. To use, dilute one part of preserved serum with seven parts of distilled water to give a 1:10 dilution.

Appendix Table 1 Proportion of infected cells (percent) before and after culture

Donor Sheep No.	Fresh	Infected cells Incubated at 37°C 4 hours	8 hours	24 hours
198	39	52	66	57
200	24	35	40	42
199	28	33	48	48
196	18	28	32	36
197	17	21	22	33
211	41	44	54	55
212	47	56	59	56
265	18	22	48	52
267	11	8	13	14

Appendix Table 2 Effects of culture on the proportion of infected cells (percent) and total counts ($10^3/mm^3$)

Donor Sheep No.	Fresh		24 hours at 37°C Stationary		Rotating		24 hours at 4°C	
	Total	TBF	Total	TBF	Total	TBF	Total	TBF
125	2.0	3	1.8	23	1.1	23	2.0	1
126	1.3	6	1.3	10	2.1	9	1.2	2
131	2.0	25	1.8	44	1.7	45	1.9	24
127	1.5	8	1.2	26	1.6	33	1.4	11
128	1.5	31	1.6	45	1.4	54	1.6	15
118	3.0	23	2.2	39	1.2	32	2.2	22
142	2.5	33	1.8	42	1.8	43	1.9	15
141	0.9	45	0.6	53	2.1	59	0.7	33
120	2.3	31	2.2	51	1.9	38	2.4	13
11	2.7	ND	ND	ND	1.0	ND	2.9	ND
12	3.2	ND	ND	ND	1.6	ND	3.0	ND
13	2.1	ND	ND	ND	1.3	ND	2.8	ND
14	2.4	ND	ND	ND	1.2	ND	2.4	ND
15	2.0	ND	ND	ND	2.1	ND	2.0	ND
16	2.0	ND	ND	ND	1.9	ND	2.0	ND
17	2.8	ND	ND	ND	0.5	ND	1.8	ND
18	3.3	ND	ND	ND	1.6	ND	2.4	ND

ND = not done

Appendix Table 3 Effects of mixing infected and uninfected blood on the proportion of infected cells

Sample No.	% TBF	
	Before culture	After 24 hours of culture
P56	< 1	13
P57	< 1	11
P59	< 1	9
P60	< 1	11
P3857	< 1	11
P3057	< 1	2
P3559	4	15
P3060	4	21
P9296	5	44
P9897	1	9
P4156	5	18
P4157	4	12
P4159	4	13
P4160	4	8

Appendix Table 4 Comparison of numbers of particles per 20 infected cells before and after culture

Sample No.	Fresh		Incubated	
	Single	Clusters	Single	Clusters
78	43	9	42	21
68	24	4	39	20
37	60	0	121	54
38	38	0	36	29
168	59	0	57	31
30	35	4	34	27
137	53	0	41	25
268	44	0	91	17
75	33	17	25	23
175	48	4	72	24
54	53	1	112	56
73	60	2	56	26
49	38	0	70	24

Appendix Table 5 Percentage of infected cells in fresh blood samples containing discrete particles

Sheep No.	Days of parasitaemia 0	1	2	3	4	5	6
77	70	100	100	80	100	90	N
86	90	100	90	80	50	50	60
118	100	80	50	50	70	100	50
121	80	60	100	60	50	70	100
124	70	80	70	100	100	60	N
125	50	90	40	80	70	50	N
126	80	80	80	50	50	80	0
142	80	50	60	80	50	70	N

Appendix Table 6 Percentage of infected cells in fresh blood samples containing clusters

Sheep No.	Days of parasitaemia 0	1	2	3	4	5	6
77	30	0	0	20	0	0	N
86	0	0	10	20	20	20	40
118	0	10	10	20	20	0	50
121	0	10	0	20	10	10	0
124	10	10	10	0	0	20	N
125	20	0	0	20	0	40	N
126	0	0	0	20	30	20	100
142	0	30	0	10	30	10	N

Appendix Table 7 Percentage of infected cells in fresh blood samples containing discrete particles and clusters

Sheep No.	Days of parasitaemia 0	1	2	3	4	5	6
77	0	0	0	0	0	10	N
86	10	0	0	0	30	30	0
118	0	10	40	30	10	10	0
121	20	30	0	20	40	20	0
124	20	10	20	0	0	20	N
125	30	10	60	0	30	10	N
126	20	20	20	30	20	0	0
142	20	20	40	10	20	20	N

N = negative sample

Appendix Table 8 Percentage of infected cells in cultured blood samples containing discrete particles

Sheep No.	Days of parasitaemia -1	0	1	2	3	4	5	6	7
77	N	30	30	30	20	0	10	0	0
86	N	10	10	30	20	10	10	10	0
118	30	30	0	0	0	20	20	0	30
121	100	0	20	10	0	0	ND	10	10
124	30	10	10	0	20	10	0	30	30
125	20	0	10	0	40	0	0	0	0
126	30	0	0	10	0	0	10	0	N
142	30	0	0	0	10	0	0	0	0

Appendix Table 9 Percentage of infected cells in cultured blood samples containing clusters

Donor Sheep	Days of parasitaemia -1	0	1	2	3	4	5	6	7
77	N	60	60	30	50	20	70	100	100
86	N	90	50	60	50	50	80	70	80
118	40	30	40	20	80	60	60	70	70
121	0	20	40	50	70	60	ND	60	60
124	40	80	80	80	60	80	80	70	70
125	80	40	60	90	60	80	90	100	100
126	50	70	70	70	80	80	80	70	N
142	60	50	80	70	80	90	90	80	80

Appendix Table 10 Percentage of infected cells in cultured blood samples containing discrete particles and clusters

Sheep No.	Days of parasitaemia -1	0	1	2	3	4	5	6	7
77	N	10	10	40	30	80	20	0	0
86	N	0	40	10	30	40	10	20	20
118	30	40	60	80	20	20	20	30	0
121	0	80	40	40	30	40	ND	30	30
124	0	10	10	20	20	10	20	0	0
125	0	60	30	10	0	20	10	0	0
126	0	30	30	20	20	20	10	30	N
142	10	50	20	30	10	10	10	20	20

N = negative sample ND = not done

Appendix Table 11 Comparison of proportion of PMN and PMN with TBF before and after attachment to glass surfaces

Sample Number	Before attachment PMN(%)	Before attachment Infected PMN(%)	After attachment PMN(%)	After attachment Infected PMN(%)
33	33	57.6	90	3.3
39	38	31.5	75	24.0
43	43	55.8	90	10.0
47	47	34.0	86	14.0
324	56	46.4	95	35.8
336	70	57.1	96	35.4
337	74	23.0	94	14.9
340	64	32.8	97	8.2
341	68	26.5	98	12.2
342	32	65.6	94	13.8
343	61	55.7	96	8.3

Appendix Table 12 Comparison of CF and IH titres

Sheep No.	Weeks post-inoculation	Titre (log_2) CF	Titre (log_2) IH	d
86	0	<1	<1	0
	1	<1	<1	0
	2	5	3	2
	3	7	5	2
	4	6	3	3
	5	7	5	2
	6	7	2	5
	7	6	4	2
	8	6	4	2
121	0	4	3	1
	1	4	2	2
	2	6	4	2
	3	6	3	3
	4	5	4	1
	5	5	3	2
	6	6	2	4
	7	6	2	4
	8	6	<1	6

$t_{17} = 6.43$ d = difference

$P < 0.001$

Appendix Table 13 Rectal temperatures (°C) of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	41.7	40.8	40.2	39.9	39.6	39.4	39.2	39.8	39.8	39.6	40.1
18	41.1	41.1	40.1	40.0	39.8	39.6	39.8	39.8	39.8	41.1	40.9
21	41.8	41.0	40.5	40.0	39.8	39.5	39.2	39.2	39.2	41.1	40.2
6	41.7	41.1	40.5	39.8	39.8	39.3	39.5	39.2	40.1	41.1	40.9
986	41.6	41.0	40.2	40.0	39.7	39.3	39.5	39.5	39.4	41.4	ND
2	41.7	41.5	40.9	39.8	39.8	39.3	39.6	40.0	40.0	40.5	40.9
992	41.9	40.1	40.1	39.6	39.3	39.3	39.3	39.3	39.4	40.5	41.5
995	41.7	40.9	40.0	39.7	39.7	39.7	39.6	40.0	39.8	41.4	ND
1000	41.5	41.0	40.2	39.8	39.7	39.3	39.7	39.9	39.9	40.7	40.9
998	42.0	40.8	40.4	40.4	39.5	39.9	39.9	39.9	39.7	40.3	ND
999	42.1	41.3	40.8	40.8	40.4	40.0	40.1	40.1	40.4	40.3	40.3

Appendix Table 14 Rectal temperatures (°C) of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	41.5	41.5	41.5	41.7	41.7	41.8	41.4	41.5	41.5	41.4	41.5
16	42.5	42.1	41.9	41.6	41.6	41.4	41.6	41.6	41.5	41.6	41.2
20	42.2	42.2	42.2	42.0	42.0	42.0	41.8	41.8	41.8	41.9	41.6
11	41.5	41.5	41.4	41.4	41.4	41.4	41.4	41.4	41.3	41.1	41.9
228	42.1	41.7	41.7	41.7	41.7	41.7	41.6	41.7	41.5	41.3	41.0
379	41.4	41.8	41.5	41.0	41.0	41.0	41.6	42.0	42.0	41.1	ND
244	41.6	42.0	42.1	41.8	42.1	41.8	42.2	42.3	42.2	41.5	39.3
248	41.3	42.0	42.0	41.4	41.5	41.4	41.6	41.6	41.6	40.8	41.0
249	41.2	41.2	41.4	41.8	41.4	41.8	41.7	41.7	41.8	41.5	41.2

ND = not done

Appendix Table 15 Total leucocytes (x 10^9/l) of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	3.3	4.2	3.8	4.3	5.6	4.0	3.3	4.0	4.1	2.5	3.2
18	6.9	8.9	10.3	8.5	9.7	9.5	10.0	8.8	6.5	4.2	4.7
21	6.5	9.6	9.5	8.7	9.0	9.4	9.4	9.2	8.1	9.0	4.1
6	6.2	7.2	11.2	11.1	10.3	9.4	10.4	10.5	9.9	5.8	7.2
986	7.1	11.0	11.9	10.9	10.0	9.4	10.0	9.7	9.7	7.0	ND
2	7.0	11.0	11.5	12.0	12.6	11.5	11.2	10.5	10.5	6.7	7.1
992	7.2	10.0	14.3	13.2	11.0	11.6	12.4	11.9	13.1	8.7	11.0
995	6.3	10.7	9.7	9.3	8.7	8.0	8.7	7.1	7.3	7.6	ND
1000	5.2	5.3	5.2	6.0	5.0	4.8	5.1	4.9	5.5	3.7	4.6
998	5.7	7.4	8.7	8.7	11.9	7.9	7.0	7.0	6.5	3.3	ND
999	3.6	6.8	7.0	9.8	7.8	6.9	8.0	8.0	8.8	4.2	ND

Appendix Table 16 Total leucocytes (x 10^9/l) of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	6.5	5.9	6.6	6.6	6.5	6.6	6.6	6.8	7.2	9.0	7.5
16	7.1	6.5	5.9	6.0	5.8	7.4	7.7	7.2	7.2	4.8	7.6
20	4.2	4.2	4.2	5.5	5.0	5.5	5.0	5.2	5.5	4.1	5.2
11	7.7	7.3	8.3	8.0	8.2	8.2	8.2	7.5	8.0	8.5	6.4
228	6.0	5.9	5.8	5.7	5.1	4.9	5.6	5.4	5.4	3.9	3.4
379	6.0	6.5	5.5	4.8	4.8	4.8	5.0	4.5	4.5	2.9	2.7
244	8.5	8.7	7.2	5.2	5.2	4.8	7.3	6.7	7.0	6.6	5.3
248	7.5	7.4	6.0	6.0	6.0	6.8	6.5	6.7	6.6	5.6	4.4
249	5.0	5.8	7.0	4.7	4.7	5.5	4.7	5.4	5.2	5.9	6.4

ND = not done

Appendix Table 17 Total granulocytes (x 10^9/l) of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	0.8	2.0	1.6	1.8	2.9	2.0	2.1	2.0	1.6	9.3	1.2
18	2.6	5.0	6.5	4.6	5.1	5.1	8.8	5.2	5.2	2.2	2.2
21	1.4	2.9	3.2	4.3	4.3	4.8	4.0	3.6	3.4	2.7	0.6
6	1.1	3.0	5.3	6.2	6.3	5.6	6.8	6.8	4.2	3.3	4.8
986	1.6	6.5	6.3	6.8	6.3	6.3	5.6	6.2	5.5	2.7	ND
2	2.2	7.9	8.4	8.4	9.3	8.9	8.3	7.9	7.9	3.6	2.8
992	1.7	4.6	8.0	6.7	7.6	5.7	6.3	6.8	6.9	3.3	5.6
995	2.9	7.2	7.1	5.8	5.9	5.9	5.1	5.2	4.2	4.7	ND
1000	2.0	2.8	3.2	4.0	3.7	3.1	3.4	3.2	3.3	2.3	2.3
998	2.5	4.4	5.6	5.6	7.6	4.9	4.8	4.8	4.1	1.7	ND
999	1.7	4.3	5.2	6.8	6.8	6.8	6.7	6.7	8.3	1.6	ND

Appendix Table 18 Total granulocytes of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	1.9	1.8	2.0	2.0	1.9	2.2	2.0	2.2	1.9	2.9	2.6
16	2.0	2.5	1.8	1.9	1.9	2.3	2.2	1.7	2.1	2.4	4.2
20	1.5	1.5	1.3	1.7	1.5	1.7	1.5	1.8	1.4	1.8	1.6
11	3.9	4.7	4.8	4.6	4.6	4.4	3.6	3.6	3.9	3.9	3.3
228	3.3	3.2	2.8	2.9	3.0	3.0	2.7	2.7	2.8	2.5	2.0
379	3.4	3.9	3.1	2.5	2.6	2.7	2.8	2.7	2.7	1.2	ND
244	5.2	5.3	4.7	4.2	3.4	3.2	4.3	3.7	3.9	3.2	2.5
248	5.0	5.1	4.1	4.1	3.8	4.8	4.9	5.0	5.0	4.5	2.7
249	2.5	3.0	3.6	3.1	2.7	2.2	2.5	2.8	2.7	3.7	3.9

ND = not done

Appendix Table 19 Total lymphocytes (x 10^9/l) of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	2.0	1.7	1.8	1.9	2.7	1.5	1.1	1.8	1.8	1.1	1.6
18	4.2	3.4	3.2	3.5	3.4	3.3	6.2	2.9	2.9	1.8	2.4
21	4.4	6.4	5.9	4.3	4.5	4.4	4.6	5.2	4.1	5.8	3.2
6	5.0	3.7	5.5	3.9	3.6	3.9	3.4	3.5	5.7	2.4	2.3
986	5.3	3.5	3.8	3.1	3.4	3.4	3.0	3.5	3.2	4.3	ND
2	3.9	2.8	2.8	2.8	2.6	2.0	2.6	1.9	1.9	2.7	3.6
992	5.5	5.3	5.7	6.5	3.0	5.5	5.8	4.9	5.6	4.9	5.2
995	3.1	2.7	2.2	2.0	2.5	1.8	1.8	2.0	2.8	2.5	ND
1000	2.8	1.7	1.4	1.7	1.3	1.1	1.5	1.2	1.7	1.0	1.6
998	3.1	2.6	2.8	2.8	3.8	2.8	1.6	1.6	2.1	1.5	ND
999	1.8	2.0	1.3	2.8	1.0	1.0	1.2	1.2	1.2	2.4	ND

Appendix Table 20 Total lymphocytes (x 10^9/l) of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	4.4	4.0	3.8	4.0	4.3	4.0	4.2	4.0	5.2	5.7	4.3
16	4.8	3.7	3.8	4.1	3.6	4.9	4.7	4.8	4.8	2.2	2.2
20	2.5	2.5	2.6	3.7	3.3	3.5	3.3	3.2	3.8	2.2	3.2
11	3.4	2.3	3.3	3.2	3.1	3.3	3.4	3.4	4.0	4.0	2.1
228	2.3	2.5	2.8	2.7	2.0	2.7	2.3	2.5	2.5	1.3	1.4
379	2.3	2.0	2.0	1.8	1.7	1.6	2.0	1.7	1.7	1.2	ND
244	3.7	3.2	2.4	2.0	1.6	1.6	2.8	2.9	3.1	3.1	2.4
248	2.4	1.7	1.4	1.7	2.0	1.7	1.6	1.6	1.6	2.1	1.6
249	2.4	2.6	3.2	2.6	2.0	2.6	2.0	2.6	2.5	2.0	2.2

ND = not done

Appendix Table 21 Total infected cells (x 10^8/l) of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	5.6	7.1	9.5	7.3	16.8	9.6	11.9	10.0	9.0	5.3	6.4
18	19.3	23.1	19.6	19.4	23.6	16.2	40.0	23.8	ND	13.9	6.6
21	10.4	14.9	17.1	13.9	13.5	22.6	18.8	23.0	15.4	15.3	12.2
6	8.7	7.2	14.5	13.3	20.6	16.0	20.8	21.0	12.9	7.5	18.0
986	9.2	17.6	16.7	16.4	15.0	17.9	24.0	24.0	30.1	5.6	ND
2	8.4	16.5	17.3	17.3	17.6	16.1	14.6	25.2	25.2	22.1	12.1
992	9.4	13.1	15.7	18.5	14.3	11.6	19.8	23.8	19.7	17.4	28.4
995	18.3	28.8	28.1	27.6	18.3	20.0	24.2	26.1	26.1	24.1	ND
1000	14.6	14.8	13.0	16.2	17.5	20.6	18.9	17.1	19.8	15.2	12.8
998	6.3	14.8	17.5	17.5	37.3	15.0	25.9	18.9	18.9	6.1	ND
999	9.3	11.6	20.7	20.7	22.3	26.9	28.2	28.2	23.5	14.0	ND

Appendix Table 22 Total infected cells (x 10^8/l) of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	12.4	11.8	13.9	11.9	12.4	11.9	9.2	8.8	14.4	7.2	11.2
16	13.5	15.0	10.6	9.0	12.2	13.3	10.0	7.9	10.0	11.5	12.9
20	8.0	8.0	7.6	10.5	5.5	11.0	10.0	8.3	9.4	8.6	8.3
11	31.6	24.8	30.7	30.7	26.5	22.1	18.9	17.8	19.5	19.6	19.2
228	25.6	21.7	19.5	16.5	15.3	13.7	11.9	13.9	13.9	10.0	3.4
379	18.1	20.0	16.5	15.4	17.3	18.6	14.4	14.4	14.4	8.6	ND
244	14.5	13.9	18.0	16.0	13.0	12.5	17.5	18.1	17.3	15.2	10.7
248	18.8	14.1	17.4	17.4	18.0	23.1	24.1	24.1	23.7	23.0	8.8
249	3.5	5.8	4.9	4.6	3.8	5.0	5.6	3.8	3.6	14.2	17.9

ND = not done

Appendix Table 23 Percentage of infected granulocytes of infected sheep injected with betamethasone

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
1	71	36	61	41	59	47	58	49	55	57	54
18	76	46	30	42	46	32	45	46	ND	64	31
21	76	52	53	33	31	47	47	64	45	57	53
6	78	24	28	21	33	29	31	31	31	23	38
986	59	27	27	25	24	28	43	39	54	21	ND
2	39	21	21	21	19	18	18	32	32	68	43
992	54	29	20	27	19	20	31	35	28	53	51
995	63	40	40	47	31	34	47	50	63	51	ND
1000	72	53	41	40	30	66	55	54	60	65	57
998	25	34	31	31	49	31	54	39	46	35	ND
999	55	27	40	30	29	40	42	42	28	89	ND

Appendix Table 24 Percentage of infected granulocytes of infected sheep injected with normal saline

Sheep No.	Hours after injection 0	1	2	3	4	5	6	7	8	24	48
991	65	59	77	63	65	54	46	46	76	30	27
16	68	60	59	47	64	60	50	36	53	40	50
20	53	53	58	62	38	65	67	59	52	48	52
11	81	53	64	67	58	50	53	49	47	50	58
228	76	68	70	57	51	51	44	52	50	40	17
379	53	51	53	62	67	69	67	53	53	72	ND
244	28	26	38	38	38	39	41	46	45	48	43
248	38	28	40	42	47	48	49	47	47	51	33
249	14	19	14	15	14	23	22	14	13	38	46

Appendix Table 25 Reciprocal antigen titres ($\log_2$) of samples collected before and after injection of betamethasone

Sample No.	Before Injection Fresh	Incubated	After Injection Fresh	Incubated
171	4	6	6	7
172	< 1	2	2	4
173	3	5	5	6
175	< 1	5	< 1	ND
176	3	5	4	ND
177	< 1	5	4	4
179	< 1	2	< 1	3
213	< 1	< 1	4	4
214	2	3	4	5

ND = not done

Appendix Table 26 Reciprocal antigen titres (log_2) of cultured samples

Sample No.	Hours of culture 0	4	8	24
195	1	ND	3	3
196	<1	1	1	1
197	1	2	3	3
198	<1	<1	<1	4
199	2	3	4	5
200	<1	4	4	4
201	2	2	3	3
211	<1	<1	<1	4
212	2	3	4	4

ND = not done

Appendix Table 27 Effects of density gradient centrifugation on the antigen titre and percentage of neutrophils and infected cells

Sample No.	Reciprocal antigen titre (log_2) Before separation	After separation	Recovery rate (%) PMN	Infected cells
185	2.5	1.5	85	62
224	2.0	3.5	90	70
228	2.0	3.0	100	68
244	2.0	3.5	100	99
248	4.0	4.0	93	97
249	2.0	3.0	85	73
250	1.0	1.0	76	51
251	1.0	1.0	74	74
379	3.0	3.5	95	86

Appendix Table 28 Reciprocal CF titre (log_2) before challenge and clinical reactions after challenge

Group	Sheep No.	Titre (log_2) before challenge	Reactions parasitaemia	Fever
Group 1	200	10	-	-
	212	10	-	-
	198	8	-	-
	199	8	-	-
	201	8	-	-
	211	7	-	-
	99	6	-	-
	161	6	-	-
	229	6	-	-
	234	6	-	-
	272	6	-	-
	165	6	-	-
	94	6	-	-
	167	5	-	-
Group 2	98	4	-	-
	124	4	-	-
	160	4	-	-
	170	4	-	-
	82	4	+	-
	126	4	+	+
	155	4	+	+
	158	4	+	+
	162	4	+	+
	169	4	-	+
Group 3	77	3	-	-
	120	3	+	+
	121	3	+	-
	125	3	+	+
	141	3	+	+
	142	3	+	+
	156	3	+	+
	159	3	+	+
	118	2	+	+
	83	<2	+	+
	85	<2	+	-

Appendix Table 29 Incubation periods of primary and secondary reaction to *C. phagocytophila*

Sheep No.	Primary	Secondary
82	3	6
83	3	6
118	3	5
120	3	4
121	3	7
125	3	6
126	4	6
141	2	4
142	4	4

Appendix Table 30 Duration (days) of fever and parasitaemia of primary and secondary reactions

Sheep No.	Primary		Secondary	
	Fever	Parasitaemia	Fever	Parasitaemia
82	8	7	0	1
83	6	9	4	5
118	6	7	2	3
120	3	5	3	5
121	6	7	0	3
125	8	7	3	5
126	10	8	3	1
141	9	6	2	5
142	14	8	5	4

Appendix Table 31 Magnitudes (mm^2) of fever and parasitaemia of primary and secondary reactions

Sheep No.	Primary		Secondary	
	Fever	Parasitaemia	Fever	Parasitaemia
82	2500	3993	75	493
83	2000	6558	1125	2840
118	1825	5223	525	2150
120	1175	4255	1225	3188
121	1850	4373	0	1480
125	3125	4733	600	2738
126	3875	4708	775	428
141	3425	4445	1175	3350
142	4100	5358	1550	2483

Appendix Table 32 Peak fever (°C) and parasitaemia (x 10^9 cells/l) of primary and secondary infections

Sheep No.	Primary		Secondary	
	Fever	Parasitaemia	Fever	Parasitaemia
82	42.3	0.9	40.3	0.8
83	42.1	3.1	41.5	2.4
118	41.7	2.8	40.6	1.3
120	41.7	3.2	41.5	1.8
121	41.8	2.9	46.0	0.2
125	42.4	2.2	40.8	0.4
126	42.1	0.9	41.3	0.1
141	42.4	3.0	42.3	1.1
142	42.1	3.4	41.4	0.7

Appendix Table 33 Neutropaenia and lymphocytopaenia of primary and secondary reactions

Sheep No.	Primary				Secondary			
	Neutropaenia		Lymphocytopaenia		Neutropaenia		Lymphocytopaenia	
	Nadir (10^3/mm^3)	Day of nadir(p.i.)	Nadir (10^3/mm^3)	Day of nadir(p.i.)	Nadir (10^3/mm^3)	Day of nadir (p.i.)	Nadir (10^3/mm^3)	Day of nadir (p.i.)
82	0.30	12	4.6	4	0.67	12	5.9	4
83	0.70	13	4.3	8	ND	ND	ND	ND
118	0.34	12	2.9	7	0.36	8	3.8	7
120	0.72	9	1.7	4	0.55	8	3.3	7
121	0.29	14	1.6	9	0.64	11	2.7	7
125	0.35	9	1.9	4	0.43	11	3.9	6
126	0.31	13	2.5	4	0.31	9	3.2	9
141	0.32	9	1.5	3	0.86	11	1.6	5
142	0.35	9	2.2	4	0.98	8	2.2	5

ND = not done p.i. = post-inoculation

Appendix Table 34 Reciprocal CF titre ($\log_2$) of pre- and post-challenge sera of sheep which did not react

Sheep No.	Before challenge	After challenge (weeks) 1	2	3
77	3	3	3	3
98	4	7	6	7
99	6	6	6	7
124	4	6	6	5
160	4	4	4	4
161	6	6	5	5
165	6	6	8	6
167	5	5	5	5
170	4	5	5	5
198	8	8	8	8
199	8	8	8	7
200	10	8	8	9
201	8	10	10	8
211	7	8	8	7
212	10	10	10	10
229	6	6	7	ND
234	6	6	7	ND
272	6	6	7	ND

Appendix Table 35 Reciprocal CF titre ($\log_2$) of pre- and post-challenge sera from sheep which reacted after challenge

Sheep No.	Pre-challenge	Weeks post challenge 1	2	3
82	4	5	7	6
83	<2	4	7	7
85	<2	5	5	6
118	2	6	6	4
120	3	6	6	4
121	3	5	6	6
125	3	6	6	6
126	4	7	6	10
141	3	6	6	6
142	3	6	6	7
155	4	4	6	6
156	3	7	6	6
158	4	6	10	5
159	3	3	7	4

ND = not done

Appendix Table 36 Total serum proteins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	51	51	53	71	68	114	104	56	53
82	82	68	62	71	82	90	91	84	87
83	65	93	88	88	88	95	88	88	103
85	74	66	72	71	97	72	75	74	69
86	66	51	53	60	67	60	67	67	58
118	55	33	75	50	56	93	52	56	64
120	66	66	95	93	99	70	78	78	70
124	75	62	71	103	71	75	105	73	ND
125	57	50	65	67	63	63	65	59	63
126	62	62	56	58	66	66	75	62	69
141	62	57	66	57	62	109	66	64	64
142	63	56	59	73	70	63	60	63	63

Appendix Table 37 Total serum proteins (g/l) of normal, uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	63	69	63	61	70	82	82	82	79
230	71	77	73	73	75	67	80	73	71
231	70	69	66	72	78	61	67	67	67
232	64	75	70	90	73	55	61	64	64
233	73	69	70	63	55	72	67	67	70
234	55	69	54	55	69	85	76	97	94
235	46	45	45	49	48	48	45	48	46
237	81	90	66	72	79	73	72	76	79

Appendix Table 38 Total serum globulins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	37	32	34	42	37	74	62	33	ND
82	53	46	ND	51	51	57	56	57	44
83	46	58	64	63	55	66	48	46	47
85	45	41	57	44	60	44	38	ND	44
86	40	40	41	42	ND	43	52	43	37
118	29	16	37	25	26	43	25	25	34
120	31	36	48	48	49	31	47	35	36
124	44	41	48	69	48	53	67	52	ND
125	27	30	38	31	29	29	37	56	37
126	29	29	28	27	30	30	45	26	35
141	36	34	40	32	35	60	38	37	36
142	34	33	34	45	47	41	34	34	37

ND = not done

Appendix Table 39 Total serum globulins (g/l) of normal, uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	37.8	38.6	35.9	36.0	39.2	50.8	51.0	51.7	43.4
230	35.5	40.8	40.1	35.0	45.0	30.8	44.0	40.1	39.0
231	38.5	37.9	35.0	43.2	54.6	37.2	41.5	39.5	43.5
232	42.2	45.0	39.9	50.2	42.3	32.4	37.2	42.2	37.1
233	48.9	42.8	44.1	34.6	22.5	44.6	42.2	ND	43.4
234	28.0	46.9	30.8	29.1	37.9	52.7	41.8	53.3	51.7
235	26.7	24.3	28.9	26.5	29.3	29.6	26.5	28.3	25.8
237	54.3	57.6	35.0	44.6	46.6	43.3	40.4	46.4	45.2

Appendix Table 40 Serum albumins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	18	18	19	29	31	39	42	23	ND
82	29	23	ND	36	31	53	36	27	31
83	20	35	24	25	33	30	41	42	56
85	29	26	16	28	37	27	38	ND	26
86	20	11	12	18	ND	17	15	24	21
118	26	17	38	26	30	50	27	31	30
120	35	31	46	46	51	39	30	33	34
124	32	21	23	34	23	22	38	21	ND
125	29	22	27	36	34	34	28	23	26
126	33	34	28	31	36	36	30	34	34
141	26	23	26	24	27	49	28	27	28
142	28	22	25	28	26	28	29	32	26

ND= not done

Appendix Table 41 Serum albumins (g/l) of normal, uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	25.2	30.4	27.1	25.6	30.8	31.2	31.0	30.3	35.6
230	35.5	36.2	32.9	38.0	30.0	36.2	36.0	32.9	32.0
231	31.5	31.1	31.0	28.8	23.4	23.8	25.5	27.5	23.5
232	21.8	30.0	30.1	39.6	30.6	22.6	23.8	21.8	26.9
233	24.1	26.2	25.9	28.4	32.5	27.4	24.8	ND	26.6
234	27.0	22.1	23.2	25.9	31.1	32.3	34.2	43.7	42.3
235	19.3	20.7	18.9	20.1	18.7	18.2	18.5	19.7	20.2
237	26.3	32.4	31.0	27.4	32.4	29.2	31.6	29.6	26.8

Appendix Table 42 Serum alpha1 globulins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	12.1	9.7	10.1	9.9	12.9	13.7	9.3	6.2	ND
82	11.5	9.5	ND	5.0	4.1	17.1	1.8	8.4	4.4
83	11.7	5.5	11.4	16.7	11.4	9.0	4.4	12.3	6.2
85	6.7	10.0	19.0	5.0	6.8	5.0	3.0	ND	5.5
86	5.4	8.7	9.0	5.4	ND	9.0	16.1	ND	7.0
118	7.7	2.0	4.5	3.0	2.8	3.7	1.0	1.1	5.1
120	2.0	2.6	14.3	4.7	3.0	0.7	6.2	4.9	4.2
124	3.8	9.3	10.6	24.7	13.5	16.5	12.6	17.5	ND
125	5.7	5.0	3.9	2.7	2.5	9.3	5.2	7.7	5.7
126	8.7	11.2	9.0	7.0	10.6	9.2	10.5	5.0	5.5
141	4.9	5.7	4.6	4.0	3.7	6.5	2.6	3.8	4.5
142	4.4	4.5	3.5	5.8	6.6	4.9	2.5	4.0	5.0

ND = not done

Appendix Table 43 Serum alpha 1 globulins (g/l) of normal, uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	2.5	2.8	1.9	3.7	4.2	4.1	4.1	4.1	4.0
230	3.6	3.9	2.9	2.9	3.8	2.0	4.0	2.9	3.6
231	3.5	3.5	3.3	4.3	5.5	2.4	4.0	3.4	4.0
232	3.8	3.0	2.8	4.5	3.7	2.8	3.1	3.2	2.6
233	2.2	4.8	2.1	4.4	1.1	4.3	4.7	ND	3.5
234	2.8	4.8	2.7	2.2	2.8	5.1	3.0	4.9	6.6
235	2.3	1.8	2.7	2.5	2.9	3.8	2.7	3.4	2.3
237	3.2	3.6	2.6	2.2	2.9	2.9	2.9	3.8	3.2

Appendix Table 44 Serum alpha 2 globulins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	9.4	8.7	6.9	13.5	8.2	11.4	13.5	8.4	ND
82	15.6	12.9	ND	8.5	9.8	16.2	10.0	11.8	14.8
83	7.2	15.6	14.1	15.8	10.6	16.2	11.4	8.8	11.3
85	14.8	16.1	10.2	10.7	17.5	9.4	6.0	ND	9.0
86	10.2	12.2	9.0	9.6	ND	8.4	10.1	ND	9.3
118	8.8	4.0	9.8	6.5	7.8	11.2	6.2	6.7	5.8
120	7.9	9.9	7.6	11.2	13.9	7.7	10.9	11.7	10.5
124	3.8	11.2	12.1	13.4	13.5	14.3	22.1	11.7	ND
125	2.3	9.0	9.8	8.7	8.2	8.2	7.2	5.9	7.6
126	6.2	6.2	5.6	10.4	4.6	5.9	12.6	4.9	8.3
141	9.3	10.3	18.9	9.1	10.5	16.4	9.9	9.6	7.0
142	9.5	9.5	8.9	11.0	10.2	9.1	8.2	9.2	8.8

ND= not done

Appendix Table 45 Serum alpha 2 globulin (g/l) of normal, uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	5.6	6.2	5.8	5.5	5.6	8.2	9.1	8.2	7.9
230	7.1	7.7	8.8	6.6	8.3	5.4	6.4	6.6	6.4
231	5.6	5.5	5.3	5.0	6.5	5.5	6.0	6.0	6.7
232	6.4	7.5	7.0	7.2	5.8	4.4	5.5	6.4	4.5
233	7.3	6.9	7.7	6.3	2.8	9.4	6.7	ND	9.1
234	3.9	4.8	4.9	3.9	6.9	6.8	5.3	5.8	6.6
235	5.1	4.1	4.1	4.9	4.3	5.8	5.9	5.8	4.6
237	8.9	8.1	6.6	7.2	7.1	8.0	6.5	7.6	9.5

Appendix Table 46 Serum gamma-globulins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	2.2	2.0	1.1	2.1	2.7	2.3	5.2	3.4	ND
82	1.6	4.1	ND	0.7	4.9	3.6	6.4	3.4	3.5
83	2.6	1.9	1.8	1.8	1.8	4.8	3.5	2.6	5.2
85	4.4	2.7	2.9	5.0	4.8	2.1	5.3	ND	1.4
86	2.4	2.0	2.7	3.0	ND	2.4	3.2	ND	1.2
118	0.5	1.3	4.3	4.5	2.2	6.5	4.2	6.1	7.6
120	4.6	7.2	7.3	7.3	5.9	4.9	6.9	7.7	5.6
124	5.2	1.9	5.0	5.1	2.8	3.0	3.1	2.9	ND
125	1.1	1.0	2.6	2.0	0.6	1.4	1.4	1.8	2.5
126	1.2	1.3	3.9	1.3	2.0	2.0	0.7	1.3	2.7
141	0.4	0.3	0.4	0.3	0.4	0.7	0.4	0.4	0.7
142	0.6	1.1	0.06	0.7	0.7	0.6	0.7	0.7	0.7

ND = not done

Appendix Table 47 Serum gamma globulins (g/l) of normal uninfected sheep injected with normal saline

Sheep No.	Weeks post injection 0	1	2	3	4	5	6	7	8
229	4.4	4.1	3.8	3.3	4.2	5.7	4.6	5.7	4.7
230	3.6	4.6	5.8	4.4	4.5	6.7	6.4	4.4	2.8
231	2.8	2.8	2.6	4.3	3.9	4.3	4.0	3.4	5.4
232	3.8	4.5	5.6	7.2	5.8	5.0	3.1	7.0	5.8
233	2.9	4.1	5.6	1.3	1.7	5.0	4.0	ND	2.8
234	1.7	4.8	4.3	3.9	2.1	6.8	3.8	5.8	8.5
235	2.3	2.3	1.8	2.0	3.4	2.4	2.3	1.9	1.8
237	6.5	6.3	3.3	4.3	3.2	4.4	3.6	4.6	4.7

Appendix Table 48 Serum beta globulins (g/l) of infected sheep

Sheep No.	Weeks post-inoculation 0	1	2	3	4	5	6	7	8
77	13.8	13.1	15.9	16.3	13.6	45.5	34.2	14.0	ND
82	24.6	19.0	ND	27.0	33.6	34.2	37.3	33.6	33.1
83	24.1	34.4	36.9	29.0	30.8	27.5	28.1	22.0	24.7
85	19.2	12.0	24.7	22.8	30.9	27.9	23.4	ND	27.6
86	22.2	16.6	20.7	24.0	ND	23.4	22.8	ND	19.5
118	12.0	8.3	17.9	10.5	13.4	21.7	13.5	10.6	15.4
120	16.5	14.2	26.5	24.2	25.7	17.5	24.3	18.9	17.6
124	30.8	18.6	19.9	15.6	18.4	20.0	29.2	19.8	ND
125	18.1	13.5	21.3	17.4	19.4	20.0	22.2	26.5	21.3
126	10.8	10.0	9.5	5.2	13.2	12.5	20.9	13.8	18.6
141	20.5	17.7	23.1	18.8	20.5	30.5	24.4	23.0	23.0
142	20.2	18.5	21.2	27.0	28.0	23.9	25.1	19.5	23.3

ND = not done

Appendix Table 49 Serum beta globulins (g/l) of normal uninfected sheep injected with normal saline

Sheep No.	Weeks post-injection 0	1	2	3	4	5	6	7	8
229	25.2	23.3	23.3	22.6	25.2	32.8	33.2	33.6	26.9
230	21.3	24.6	22.6	21.8	28.5	16.8	27.2	24.1	26.3
231	26.6	26.2	23.8	29.5	38.2	25.0	27.5	26.8	27.5
232	28.2	30.0	28.7	31.5	26.3	20.4	25.6	25.6	24.3
233	28.5	26.9	28.7	22.7	17.1	25.9	26.8	ND	28.0
234	19.8	27.4	18.9	19.3	26.2	34.0	29.6	36.9	41.4
235	17.0	16.7	17.6	19.6	18.7	17.8	15.8	17.3	16.6
237	35.6	39.6	21.8	31.0	33.2	28.5	27.4	30.4	34.8

ND = not done

Appendix Table 50 Time (weeks) on which peak CF titres occurred on the IgM and IgG fractions

Sheep No.	Week of peak titre IgM	IgG
155	4	3
156	2	4
157	2	3
158	4	3
159	2	6
160	3	8
161	2	4
82	2	6
86	2	2
124	4	7

Appendix Table 51 Reciprocal CF titres (log_2) of sera before and after treatment with 2-mercapthoethanol

Sheep No.	Before Treatment Weeks post-inoculation									After Treatment Weeks post-inoculation								
	0	1	2	3	4	5	6	7	8	0	1	2	3	4	5	6	7	8
155	<2	<2	5	5	5	4	5	5	5	<2	<2	4	4	4	4	5	ND	ND
156	<2	<2	7	7	8	8	6	7	6	<2	<2	6	6	4	4	4	4	6
157	<2	<2	10	9	7	8	7	4	4	<2	<2	3	4	7	6	6	4	4
158	<2	<2	7	8	7	7	7	6	5	<2	<2	5	6	6	6	6	5	5
159	<2	<2	7	8	7	6	6	5	4	<2	<2	6	7	7	6	5	4	4
160	<2	<2	6	ND	4	5	4	4	3	<2	<2	5	ND	4	4	4	<2	3
161	<2	3	7	8	6	5	6	3	4	<2	<2	6	6	6	4	4	<2	3
162	<2	3	7	7	7	6	6	6	6	<2	<2	2	3	4	3	ND	ND	3
165	<2	6	8	7	6	6	6	4	4	<2	<2	5	4	5	5	5	4	4

ND = not done

Appendix Table 52 Areas of migration*of leucocytes of normal sheep injected with normal saline (Group 1) when cultured without antigen

Days PI	Sheep Number 77	86	100	101	102	103	104	229	230	Mean	s.d.
0	84	111	96	66	155	89	127	174	132	115.44	35.57
4	43	108	100	66	ND	73	170	139	123	102.75	41.55
7	62	102	122	67	78	70	53	127	143	91.56	32.64
10	62	154	162	66	77	77	125	ND	ND	103.29	42.75
14	100	164	79	100	80	58	67	162	190	111.11	48.25
17	75	106	94	63	70	ND	ND	200	230	119.71	67.26
21	60	144	114	58	60	57	62	ND	ND	81.50	32.24
24	182	48	89	60	59	91	66	ND	ND	85.14	45.54
28	174	120	85	78	61	67	71	136	169	106.78	42.27
35	205	159	97	52	70	60	78	132	150	111.44	52.62
42	118	129	107	60	121	58	67	137	147	104.89	34.07
49	164	71	69	57	64	104	63	97	182	96.78	46.19
56	206	150	70	62	ND	72	58	114	164	112.00	55.76

ND = not done *mm^2 pre = pre-injection PI = post injection

s.d. = standard deviation

Appendix Table 53 Areas of migration*of leucocytes of normal sheep injected with normal saline (Group 1) cultured with antigen

Days PI	Sheep Number 77	86	100	101	102	103	104	229	230
0	87	114	59	90	163	86	117	134	144
4	41	89	80	101	ND	94	151	144	135
7	58	98	65	146	82	73	59	130	124
10	58	161	57	165	76	67	113	ND	ND
14	90	157	90	89	75	60	67	150	170
17	69	92	55	94	62	ND	ND	186	215
21	66	160	61	110	68	62	57	ND	ND
24	179	52	62	88	64	90	66	ND	ND
28	167	110	82	82	65	72	77	130	157
35	192	170	52	97	65	60	79	122	168
42	100	124	61	111	122	54	69	160	145
49	162	69	62	79	65	113	69	90	182
56	199	155	56	66	ND	68	50	114	164

ND = not done

PI = post-injection

Appendix Table 54 Areas of migration*of leucocytes of sheep infected with C. phagocytophila (Group 2) when cultured without antigen

Days post inoculation	Sheep Number 78	79	82	83	85	98	99	228
0	79	37	145	139	106	247	240	168
4	54	49	63	98	32	70	17	88
7	65	49	40	56	44	115	50	82
10	65	49	53	41	38	45	34	96
14	18	54	35	65	47	63	49	111
17	24	59	22	52	90	109	65	137
21	44	74	96	120	62	165	66	158
24	86	88	82	79	53	53	51	130
28	122	99	58	87	88	47	50	109
35	106	125	65	63	74	64	42	81
42	96	178	40	55	62	92	94	76
49	62	106	61	49	72	56	61	97
56	67	156	67	ND	153	112	170	ND

*mm^2

Appendix Table 55 Areas of migration*of leucocytes of sheep infected with C. phagocytophila (Group 2) when cultured with antigen

Days p.i.	Sheep Number 78	79	82	83	85	98	99	228
0	72	26	160	150	100	265	226	167
4	35	40	64	97	27	33	17	71
7	48	22	42	42	16	69	38	78
10	48	22	52	33	21	30	33	83
14	9	21	32	52	48	56	40	88
17	19	62	25	49	74	53	47	98
21	58	65	80	55	27	127	62	109
24	90	64	20	33	31	24	23	82
28	87	54	24	65	35	36	38	56
35	98	77	11	41	47	54	43	58
42	35	178	26	36	55	92	96	41
49	31	69	41	52	40	56	54	74
56	33	139	47	ND	96	96	122	ND

ND = not done

p.i. = post inoculation

Appendix Table 56 Migration areas* of leucocytes of normal sheep injected with normal saline (Group 3) when cultured without antigen

Sheep No.	Days post injection 0	1	2	3	4
120	91	96	86	88	92
125	69	67	63	71	71
126	66	70	58	75	54
127	79	67	101	100	100
128	180	171	179	154	174
141	70	89	88	78	83
229	174	182	192	160	139
230	137	140	134	130	123

*mm^2

Appendix Table 57 Migration areas* of leucocytes of normal sheep injected with normal saline (Group 3) when cultured with antigen

Sheep No.	Days post-injection 0	1	2	3	4
120	65	71	71	62	58
125	92	99	94	91	97
126	65	64	61	88	46
127	78	73	91	95	95
128	202	180	171	153	185
141	74	95	94	78	87
229	134	144	200	165	144
230	144	135	140	136	135

Appendix Table 58 Migration areas* of leucocytes of infected sheep (Group 4) cultured without antigen

Sheep No.	Days post inoculation 0	1	2	3	4
118	87	86	60	52	33
120	109	120	86	29	33
121	110	47	28	44	40
124	80	86	60	44	33
125	85	85	88	44	36
126	112	150	140	34	43
141	118	100	98	21	29
142	81	89	49	35	33

Appendix Table 59 Migration areas* of leucocytes of infected sheep (Group 4) cultured with antigen

Sheep No.	Days post inoculation 0	1	2	3	4
118	88	97	62	57	37
120	110	111	91	27	35
121	110	45	28	55	51
124	85	77	60	44	33
125	88	93	86	42	40
126	101	103	105	32	39
141	117	109	116	30	32
142	82	86	48	35	34

*mm^2

Appendix Table 60 Migration areas*of leucocytes of normal, uninfected sheep (Group 6) cultured with or without LMIF

Sheep No.	Migration area				
	Controls	LMIF C[a]	LMIF 1	LMIF 2	LMIF 2A[a]
234	45	ND	ND	45	10
235	90	79	52	75	49
236	80	92	7	71	10
237	80	75	41	75	30
238	82	72	10	100	21
239	101	115	5	100	40
240	120	110	ND	120	28
241	66	ND	5	45	26
243	230	250	17	240	45
Mean	99.33	113.29	19.57	96.78	28.78
s.d.	53.27	62.59	19.11	59.21	14.02

[a] antigen added during culture of cells

ND = not done

*mm^2